REMARKS

Status of the application

Claims 1-31 are pending in the application. Claims 10-15, 17, 19, and 23-26 were withdrawn from consideration as directed to non-elected inventions. Claims 1-9, 16, 18, 20-22 and 27-31 were under examination and stand rejected.

With entry of the instant response, claim 18 has been canceled without prejudice. Claims 1-3, 9, 16, 20, 22, and 27-31 have been amended. Specifically, Claims 1-3 have been amended to specify that the disorder to be treated is depression or drug addiction selected from the group consisting of nicotine, alcohol, opiate, amphetamine, methamphetamine and cocaine. Similarly, claims 16 and 27 have been amended to specify these addictive disorders. Support for these amendments can be found in the specification, e.g., at page 18, last full paragraph.

In addition, claims 1-3, 20, 27 and 29 have been amended to specify that an antagonist of mGluR2/3 and an antagonist of mGluR5 are both administered to the subject in the claimed therapeutic methods. Support for the amendment is replete in the specification, e.g., at page 4 (first full paragraph); page 23 (middle paragraph); and Example 3 (pages 64-79). Dependent claims 9, 22, 28 and 31 have been accordingly amended to recite the two specific antagonist of mGlu2/3 and mGlu5, respectively, LY341495 and MPEP. In addition, claims 22, 28 and 31 are amended to insert the chemical name of LY341495, and claim 30 is amended for improved clarity.

Applicant submit that the claim amendments presented herein do not introduce new matter. Unless otherwise noted, the claim amendments have been made to better present Applicants' inventions and should not be construed as acquiescence of any ground of rejections. The following remarks address issues raised in the instant Office Action.

Informalities in the Specification

The informalities in the text of the specification at page 69 as noted by the Examiner and at several other pages have been corrected by the amendments to the specification presented above.

Claim Objections

1. Claims 22, 28 and 31 have been amended herein to replace the recitation of LY341495 with its chemical name, as suggested by the Examiner.

2. The typos in original claims 16 and 18 have already been corrected by Applicants in the preliminary amendment dated March 10, 2005.

Claim rejections under 35 USC §§ 112 (2nd paragraph) and 102

Claim 18 was rejected as allegedly being indefinite in failing to recite specific steps. The claim has been canceled herein. The rejection is therefore moot.

Claim rejections under 35 USC § 112 (1st paragraph)

A number of rejections were raised in the office action alleging that the pending claims are not enabled. These rejections are addressed in turn below.

1. Claims 1-4, 16, 18, 27 and 28 were rejected on the ground that the disclosure does not enable the claimed invention with respect to all metabotropic glutamate disorders and all addictive disorders. The Examiners acknowledges that the specification is enabling for methods of treating addiction of nicotine, alcohol, opiate, amphetamine, methamphetamine and cocaine. Similarly, claims 8, 16, 18 and 29-31 were rejected on the ground that the specification does not enable methods of treating non-drug induced depression and anxiety. The Examiner takes the view that the

disclosure only enables treatment of drug-induced depression and anxiety.

Applicants do not agree with the Examiner's assertion that the subject disclosure only enables treatment of the noted specific drug additions. Nevertheless, in an effort to advance prosecution of the patent application, Applications have herein amended claim 1 to specify that the metabotropic glutamate disorder to be treated is depression or one of these specific addiction disorders. Therefore, this aspect of the present rejection should be withdrawn.

Applicants respectfully traverse the rejection of claims 8, 16, and 29-31, and disagree with the Examiner's view that the subject disclosure only enables treatment of drug-induced depression and anxiety. The Examiner correctly noted that the specification has exemplified treatment of drug-induced depression and anxiety. However, as detailed below, it does not follow that the present invention does not enable treatment of non-drug-induced depressions.

To begin, Applicants note that what is exemplified in a patent specification should not be equated with teachings of the specification. It is well established that an applicant should not be limited to the specific embodiments identified in the specification when other operable embodiments may be discovered with only routine experimentation using the teaching of the specification (see, e.g., *In re Goffe*, 191 USPQ 429, 431 (CCPA 1976) ["To demand that the first to disclose shall limit his claims to what he has found will work or to materials which meet the guidelines specified for 'preferred' materials in a process such as the one herein involved would not serve the constitutional purpose of promoting progress in the useful arts"]).

In the instant case, the specification exemplified the claimed invention by showing treatment of drug induced depression with co-administration of mGluR2/3 receptor antagonist and mGluR5 antagonist (e.g., Example 3 at page 64). The specification further noted "the known neurobiological similarities mediating drug- and non-drug-induced depressions", and explicitly taught that the co-administration of

mGluR2/3 antagonist and mGluR5 antagonist can be "efficacious for treating non-drug-induced depressions" (see, e.g., page 20, lines 2-4). At page 20, the specification cited a number of prior art references which taught the biological similarities between drug-induced and non-drug-induced depressions. The specification then further noted that:

> Additional observations further support the conclusion that results presented in Example 1 related to depression-like symptoms of withdrawal of an addictive substance, establish that antagonists of mGluR2 and/or mGluR3 can be used to effectively treat non-drug-induced depressions, as well. First, it has been shown that co-administration of the selective serotonin reuptake inhibitor fluoxetine and the serotonin-1A receptor antagonist p-MPPI, a clinically proven antidepressant drug treatment, reverses the depression-like aspects of both nicotine and amphetamine withdrawal (Harrison et al., (2001); incorporated in its entirety by reference). Second, as shown in Example 4, co-administration of the selective serotonin reuptake inhibitor paroxetine and the serotonin-1A receptor antagonist p-MPPI, another clinically proven antidepressant drug treatment, also reversed amphetamine withdrawal. Third, bupropion, another clinically proven antidepressant treatment, reverses the depression-like aspects of nicotine withdrawal (Cryan, J. F., et al., Psychopharmacology, 168, 347-358 (2003); Example 5). Thus, clinically proven antidepressant treatments reverse the depression-like aspects of drug withdrawal in the model presented in Examples 1 and 4. Therefore, it can be inferred that a treatment (e.g., mGluR2/3 antagonist) that normalized thresholds in the model, would be a clinically effective treatment. Further, the reversal of both amphetamine and nicotine withdrawal by the same antidepressant treatment indicates that there are commonalities in various types of depression, independent of what the depression-induction mechanism is and/or the primary site of action of the drug of abuse (nicotinic receptor for nicotine, monoaminergic transporters for amphetamine). [page 20; emphasis added]

Thus, upon reading the subject specification, a skilled artisan was taught how to treat drug-induced depressions as presently claimed. The skilled artisan also knew that the underlying neurobiological mechanism of depressions, drug-induced or

otherwise, are quite similar. The skilled artisan was further explicitly taught by the subject specification that the co-administration of mGluR2/3 antagonist and mGluR5 antagonist would also be efficacious for treating non-drug-induced depressions. In light of such teachings, it would readily apparent that the subject specification also enabled treatment of non-drug-induced depressions. This is because non-drug induced depression disorders were themselves well known. One certainly needs not to expend undue experimentation, if any experimentation, to ascertain a patient who suffers from non-drug induced depression. In addition, the specification has provided detailed guidance on treating drug-induced depressions, such as the compounds to be used, compound concentrations, and administration procedures (see, e.g., Examples 3). A skilled artisan can easily adopt and modify, if necessary, such teachings in practicing the claimed invention on subjects suffering from non-drug induced depressions. As clarified herein, there are certainly no scientific gap or technological hurdles between the different types of depressions that require undue experimentation to overcome.

For the reasons stated above, Applicants submit that the subject specification enables the treatment of drug-induced depressions as well as non-drug induced depressions as currently claimed. Withdrawal of the present rejection is accordingly respectfully requested.

2. Claims 1-8, 16, 18, 20, 21, 27, 29 and 30 were rejected on the ground that the specification does not enable all antagonists of the recited mGluRs. The Examiner asserts that the disclosure only enables the use of several specific antagonists exemplified in the specification, e.g., LY341495, MPEP, MK-801, NBQX disodium, LY314582 and LY354740. In essence, the rejection is based on the Examiner's belief that it requires undue experimentation to find other antagonists of mGluR2, 3, and 5 that are currently not known. This rejection is respectfully traversed for the reasons

stated below.

First, Applicants note that the reasoning set forth in the Office Action concerning unknown mGluR antagonists would be more relevant if Applicants' invention is a generic claim directed to compositions of mGluR antagonists. However, the claimed invention relate to methods of using mGlu2/3 and mGlu5 antagonists in the treatment of disorders mediated by or associated with metabotropic glutamate (e.g., drug addiction or depression). As such, the question of enablement here is whether the skilled artisan can practice the claimed methods based on the subject disclosure. Enablement of the claimed methods does not turn on whether Applicants are in possession of all likely antagonists of the mGlu receptors recited in the claims.

As the Examiner can readily appreciate, patentability of the claimed invention resides on the discovery by the present inventors that antagonism of both group I and group II receptors leads to additive effects that are unknown and unexpected in the art. So long as the employed antagonists can perform the desired functions (i.e., antagonize mGlu2/3 and mGlu5), their exact nature or structures are not material to the practice of the claimed invention. It would be entirely unreasonable and incorrect to require Applicants to disclose all likely species of a specific material or agent for practicing a claimed process. Otherwise, since there can always be more species of a given material or agent that are not currently known, no one would ever be able to patent a process which recites the use of a material or agent in generic term.

Turning to the specific case, the disclosure provided in the specification undoubtedly enabled a skilled artisan to practice the claimed methods. The specification has disclosed a representative number of antagonists of the mGlu receptors (see, e.g., pages 15-16). The specification also provides general teachings as well as specific exemplifications of using such antagonists in a subject to alleviate symptoms associated with metabotropic glutamate disorders (see, e.g., Examples in the specification). This is all that is required in order to enable the claimed invention.

For the sake of argument, even assuming one needs to use mGluR antagonists not specifically exemplified in the specification or not presently known in the art, there would nonetheless be no undue experimentation required. The test of enablement of in such a case is whether the unknown antagonists can be routinely examined and determined for applications in the practice of the claimed methods. To this end, the Examiner is reminded that routine experimentation is not undue. The courts have held that in production of variants of an exemplified species, even a considerable amount of individualized screening with its inevitable proportion of negative results is expected and considered routine by one of ordinary skill. See, e.g., *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). See also MPEP 2164.06(b). In addition, it is well settled in the law that the test of enablement "is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *In re Wands*, 858 F.2d 737 (Fed. Cir. 1988) (citing In re Angstadt, 537 F.2d 489, 502-04, 190 USPQ 214, 217-19 (CCPA 1976)). Time and expense are merely factors in this consideration and are not the controlling factors. *United States v. Telectronics Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988), cert. denied, 490 U.S. 1046 (1989).

In the present case, the procedures needed to ascertain additional mGluR antagonists for practicing the claimed methods are all routine, and no undue experimentation is required. For example, for any candidate mGluR antagonist, it only entails routinely practiced methods (e.g., those described in the subject specification) to examine its effect on a metabotropic glutamate disorder. Only standard pharmaceutical procedures are required to ascertain appropriate dosages of the mGluR antagonist. Such routine experimentation is certainly not undue as alleged in the Office Action.

For all the reasoning and clarifications presented above, Applicants submit that

the claimed invention is enabled with respect to the recitation of mGluR antagonist. Withdrawal of the instant rejection is therefore requested.

Claim rejections under 35 U.S.C. §102

Claims 1-5, 20 and 21 were rejected rejected under 35 USC 102(b) as allegedly anticipated by Fundytus et al. (British J. Pharmacol. 120:1015-20, 1997). Claims 1-5, 7, 9, 20 and 21 were rejected rejected under 35 USC 102(b) as allegedly anticipated by Chiamulera et al. (Nat. Neurosci. 4:873-4, 2001). Claims 1-8, 20, 21 and 29 were rejected under 35 USC 102(b) as allegedly anticipated by Adam et al. (US Patent No. 6407094). Claims 1-8, 20, 21 and 29 were rejected under 35 USC 102(e) as allegedly anticipated by Corsi et al. (US patent application 2003/0195139).

Applicants do not agree with the reasoning set forth in the office action in rendering these rejections. In addition, the claims as currently amended specify the use of both an antagonist of mGluR2/3 and an antagonist of mGluR5 in the treatment of a metabotropic glutamate disorder. None of the cited references teaches or suggests the use of such a combination of mGluR antagonists. As such, the presently claimed methods are clearly distinguishable over the cited art. Accordingly, the instant rejection should be withdrawn.

Claims rejection under 35 U.S.C. §103

Claims 16, 18 and 27 were rejected as allegedly being obvious over Adam et al. in view of Corsi et al. Claims 22, 27 and 28 were rejected as allegedly being obvious over Chiamulera et al. in view of Adam et al. Claims 30 and 31 were rejected as allegedly obvious over Bear et al. (US Patent No. 6916821) in view of Adam et al.

As noted above, the presently claimed invention is directed to methods of using both an antagonist of mGluR2/3 and an antagonist of mGluR5 in the treatment of metabotropic glutamate disorders. For the reasons stated below, Applicants

respectfully traverse each of these rejections to the extent that they may be applied to the presently amended claims.

First, Group I glutamate receptors such as mGlu5 are located postsynaptically. Blockade of these receptors results in decreased glutamate signaling. On the other hand, Group II receptors such as mGluR2/3 are located presynaptically. Blockade of these receptors results in increased release of glutamate and thus increases glutamate signaling. Such different biological activities of mGluR2/3 and mGluR5 are well known in the art, e.g., as described in the attached pre-priority publications, Kilbride et al., Eur. J. Pharmacol. 356:149, 1998; and Vignes et al., Neuropharmacol. 34:973-82, 1995; and Schoepp, Neurochem. Int., 24:439, 1994. Thus, contrary to the Examiner's assertions, it would be simply counter intuitive to co-administer antagonists of mGlu receptors of Group I and Group II. Therefore, one certainly would not be motivated to combine the teachings of the references cited in the Office Action.

In addition, even assuming for the sake of argument that one wanted to co-administer an mGluR2/3 antagonist and an mGluR5 antagonist, he surely could not have any reasonable expectation that such an approach would lead to better therapeutic effect. To the contrary, he would definitely be concerned that, due to the localization of these receptors and their apparent opposing effects on glutamate signaling, co-administration of mGluR2/3 and mGluR5 antagonists would likely antagonize each other's effects to the extent that their effects are neutralized. He certainly would have no reason to expect that co-administration of mGluR2/3 and mGluR5 antagonists would provide the surprising and unexpected additive effects that were first observed by the present inventors (see, e.g., page 10 and Example 3).

With due respect, Applicants note that the instant rejection is a typical example of "hindsight-based obviousness analysis." The alleged obviousness stems from nothing but the prohibited hindsight gleaned from the subject disclosure.

To summarize, it is readily apparent that the currently rejected claims (i.e.,

claims 16, 18, 22, 27, 28, 30 and 31) as well as the other claims pending in the application were not and could not be obvious over the cited art. Applicants accordingly respectfully request that these rejections be withdrawn.

CONCLUSION

In view of the foregoing, Applicants respectfully submit that the claims now pending in the subject patent application are in condition for allowance, and notification to that effect is earnestly requested. If a telephone conference would expedite prosecution of this application, please telephone the undersigned attorney at 858-784-2937.

The Director is hereby authorized to charge our Deposit Account No. 19-0962 in the event that there are any charges associated with the present Response or any Response in connection with this application.

Respectfully submitted,

September 4, 2007
Date

Hugh Wang, Ph.D., Reg. No. 47,163

Enclosures: 3 publications referenced herein

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European Journal of Pharmacology 356 (1998) 149–157

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Presynaptic inhibitory action of the group II metabotropic glutamate receptor agonists, LY354740 and DCG-IV

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Received 5 March 1998; revised 30 June 1998; accepted 10 July 1998

Abstract

Electrophysiological studies were carried out on the presynaptic inhibitory action of the group II metabotropic glutamate (mGlu) receptor agonists (+)-2-aminobicyclo[3.1.0]hexane-2-6-dicarboxylic acid (LY354740) and (2*S*,1′*R*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) in three paths of the rat hippocampus, the medial and lateral perforant path to the dentate gyrus, and the Schaffer collateral/commissural path to CA1. LY354740 caused a dose-dependent reversible inhibition of the field excitatory postsynaptic potential (EPSP) in the medial and lateral perforant paths, with an EC_{50} of 115 ± 16 nM and 230 ± 58 nM, respectively. Maximal inhibition by LY354740 was much greater in the medial path (about 80%) than in the lateral path (about 50%). No inhibition was observed in CA1. A presynaptic inhibition was confirmed by LY354740 inducing dose-dependent changes in paired-pulse depression/facilitation. DCG-IV had a similar action to LY354740, but with a lower potency. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Glutamate receptor, metabotropic; Perforant path; Paired-pulse

1. Introduction

The mediation of presynaptic inhibition by metabotropic glutamate (mGlu) receptors was first established in studies in which 1*S*,3*R*-aminocyclopentane-1,3-dicarboxylic acid (*trans*-ACPD) was shown to reversibly depress excitatory synaptic transmission in hippocampal CA1 (Baskys and Malenka, 1991; Desai and Conn, 1991; McGuinness et al., 1991; Pacelli and Kelso, 1991). A large number of studies have subsequently confirmed the presence of presynaptic mGlu receptors in many other areas of the brain, such as the hippocampal dentate gyrus (Macek et al., 1996; Bushell et al., 1996), neocortex (Sladeczek et al., 1993; Burke and Hablitz, 1994) and the striatum (Lovinger, 1991, Calabresi et al., 1992). Strong evidence that the inhibition of excitatory synaptic transmission was mediated presynaptically was shown by several lines of evidence. Firstly, inhibition by mGlu receptor agonists occurred without postsynaptic changes (Lovinger, 1991; Baskys and Malenka, 1991; Calabresi et al., 1992; Glaum et al., 1992; Lovinger et al., 1993; Burke and Hablitz, 1994); secondly, mGlu receptor activation reduced the AMPA- and NMDA-receptor mediated excitatory synaptic transmission with a similar potency (Baskys and Malenka, 1991; Lovinger, 1991; Pacelli and Kelso, 1991). Thirdly a change in paired-pulse facilitation or depression (indicative of a presynaptic modulation of transmitter release), was evoked by mGlu receptor agonists. Thus paired-pulse facilitation in CA1 was enhanced by mGlu receptor agonists (Baskys and Malenka, 1991; Burke and Hablitz, 1994; Gereau and Conn, 1995; Manzoni et al., 1997) and paired-pulse depression in the medial perforant path of the dentate gyrus being reduced by mGlu receptor agonists (Kahle and Cotman, 1993; Brown and Reymann, 1995).

Recent studies using a number of agonists selective for mGlu receptor group subtypes have shown the widespread presence of presynaptic group II mGlu receptors. In the hippocampus, a number of group II mGlu receptor selective agonists have been found to inhibit excitatory synaptic transmission, including (1*S*,3*S*)-1-aminocyclopentane-1,3-dicarboxylic acid {(1*S*,3*S*)-ACPD} (Vignes et al., 1995) and (2*S*,1′*R*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)-glycine (DCG-IV) (Yokoi et al., 1996) in young CA1; (2*S*,1′*S*,2′*S*)-2-carboxycyclopropyglycine (LCCG-1)

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PII: S0014-2999(98)00526-3

(Ugolini and Bordi, 1995) and DCG-IV (Macek et al., 1996; Huang et al., 1997) in the medial perforant path of adult dentate gyrus, and DCG-IV (Macek et al., 1996; Bushell et al., 1996) and (1*S*,3*S*)-ACPD (Bushell et al., 1996) in the lateral perforant path of neonatal (Bushell et al., 1996) and adult (Macek et al., 1996) dentate gyrus.

(+)-2-Aminobicyclo[3.1.0]hexane-2-6-dicarboxylic acid (LY354740) is a recently synthesised high affinity efficacious and selective group II mGlu receptor agonist. (Bond et al., 1997; Monn et al., 1997; Schoepp et al., 1997a,b). LY354740 suppressed forskolin-stimulated cyclic 3′,5′-adenosine monophosphate (cAMP) formation at group II mGlu receptor with nanomolar potency, but had little or no agonist or antagonist action at group I mGlu receptor or group III mGlu receptor. The agent has potentially important clinical uses—it was found to prevent anxiety in the elevated plus maze and also prevent ACPD-induced limbic seizures. Moreover, it is orally active.

In the present study, we have investigated the presynaptic inhibitory action of LY354740 in the medial and lateral

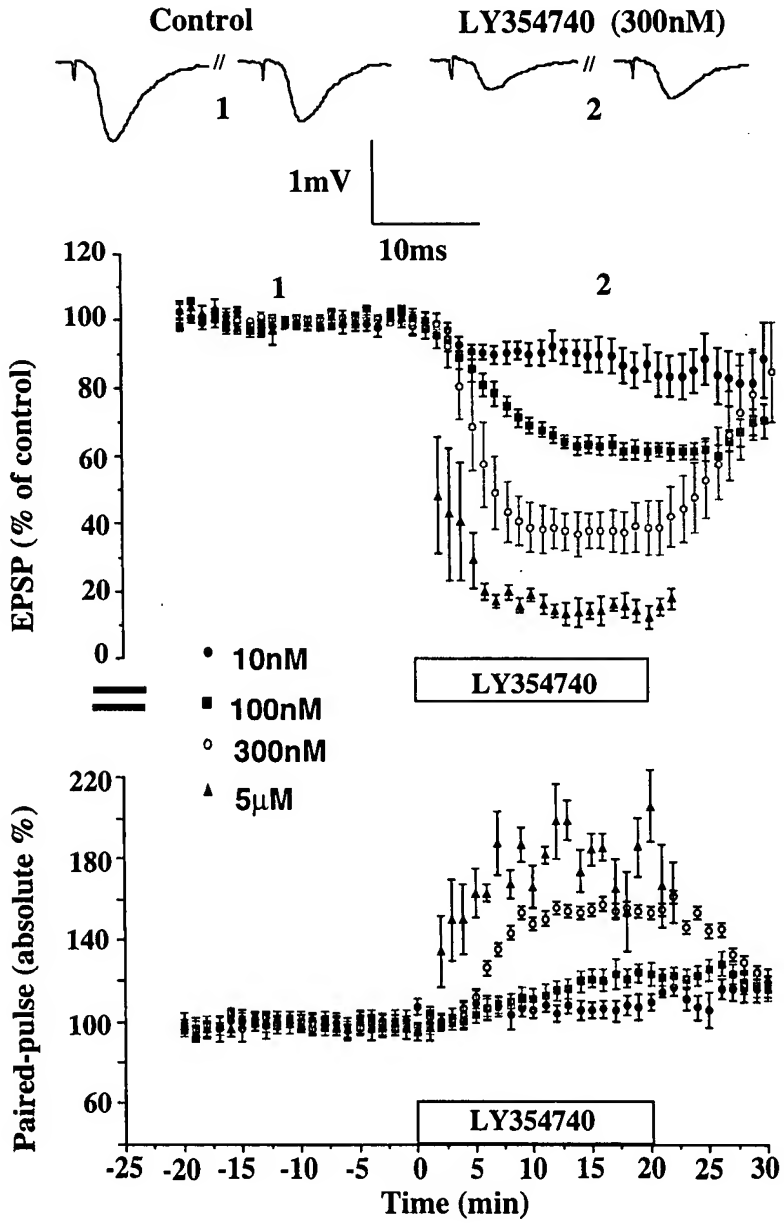


Fig. 1. LY354740 evokes a dose-dependent inhibition of excitatory synaptic transmission in the medial perforant path of the dentate gyrus in vitro. Upper graph, following a stable baseline for 20 min, perfusion of LY354740 at doses of 10 nM, 100 nM, 300 nM and 5 μM resulted in an increasing inhibition of the field EPSPs. Lower graph, 10 nM, 100 nM, 300 nM and 5 μM LY354740 caused an increasing reduction in paired-pulse depression accompanying the inhibition of the EPSP. The original traces show pairs of EPSPs in control and following application of 300 nM LY354740.

perforant path of the hippocampal dentate gyrus, and also CA1 hippocampus, comparing its action with the well established group II mGlu receptor agonist, DCG-IV.

2. Materials and methods

All experiments were carried out on hippocampal slices obtained from Wistar rats (50–70 g) (BioResources Unit, Trinity College, Dublin, Ireland). Slices were obtained as described previously (Huang et al., 1997). Briefly, the brain was rapidly removed after decapitation and placed in cold (5°C) oxygenated (95% O_2, 5% CO_2) artificial cerebro-spinal fluid (ACSF) containing in mM: NaCl, 120; $NaHCO_3$, 26; NaH_2PO_4, 1.25; KCl, 2.5; Mg_2SO_4, 2; $CaCl_2$, 2; glucose, 10). Hippocampal slices (350 μM) were cut using a Campden vibroslice (Campden Group Instruments, London, UK) and transferred immediately to an incubation chamber, maintained at room temperature, for a period of at least 60 min. Single slices were then transferred to a submersion type recording chamber at 30–31°C.

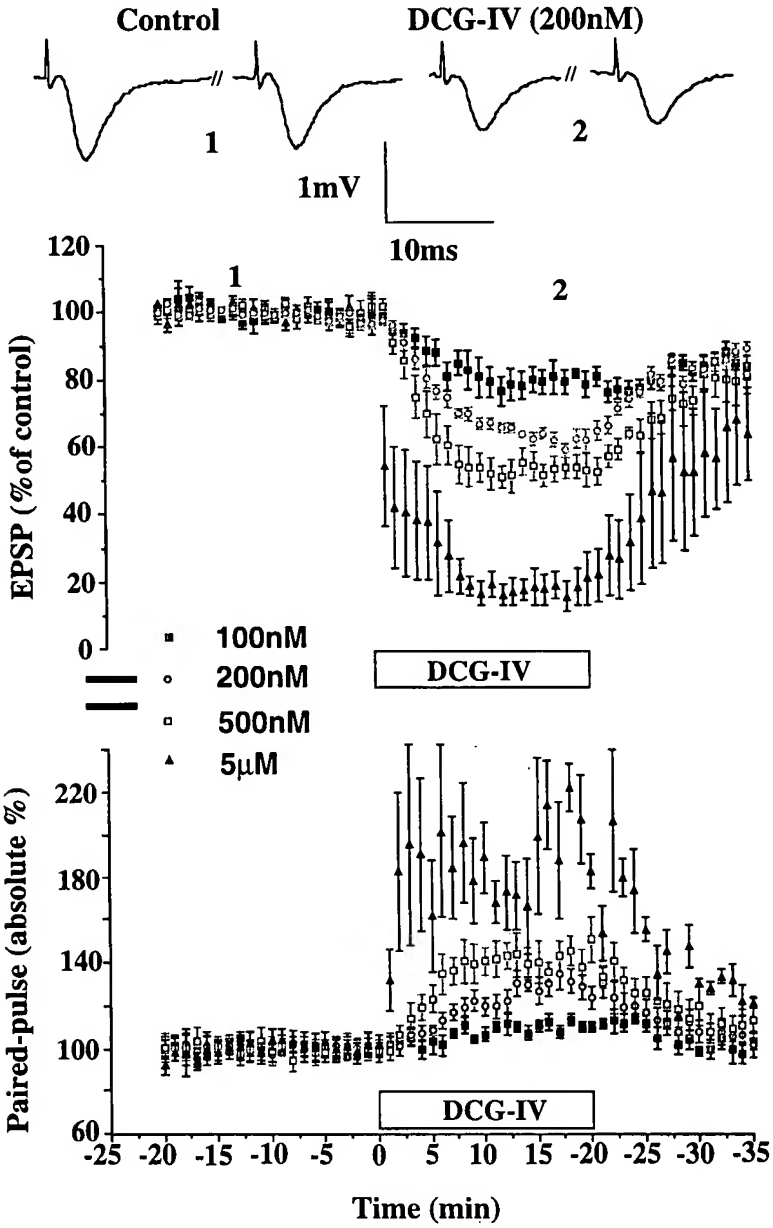


Fig. 2. DCG-IV evokes a dose-dependent inhibition of excitatory synaptic transmission in the medial perforant path of the dentate gyrus in vitro. Upper graph, following a stable baseline for 20 min, perfusion of DCG-IV at doses of 100 nM, 200 nM, 500 nM and 5 μM resulted in an increasing inhibition of the field EPSPs. Lower graph, 100 nM, 200 nM, 500 nM and 5 μM DCG-IV caused an increasing reduction in paired-pulse depression accompanying the inhibition of the EPSP. The original traces show pairs of EPSPs in control and following application of 200 nM DCG-IV.

Field excitatory postsynaptic potentials (EPSP) were recorded using standard glass electrodes filled with ACSF. Both recording and stimulating electrodes were placed in either the middle or outer third of the molecular layer of the dentate gyrus in order to stimulate and record from either the medial or lateral perforant path, respectively, and in the Schaffer collateral/commissural path in the stratum radiatum of CA1. Test EPSPs were evoked using a Grass S48 stimulator (0.0166 Hz, pulse width 0.1 ms) via a bipolar insulated tungsten wire electrode, adjusted to give about 30% of the maximal response (~ 1 mV). EPSP amplitude was measured using Maclab Scope, version 3.4. Paired-pulse stimulation (interstimulus interval of 40 ms) was applied in all experiments. For each pair, the amplitude of the second EPSP was divided by the first and multiplied by 100 to give the paired-pulse 'percentage'. A paired-pulse percentage of less than 100 was indicative of paired-pulse depression; a percentage greater than 100 was indicative of paired-pulse facilitation. The effect of LY354740 in the lateral perforant path and CA1 are presented in this way, in the text and in Figs. 3 and 5. However, the effects of LY354740 in the medial perforant path and also DCG-IV in the medial and lateral perforant path are represented in the text and Figs. 1, 2 and 4 as the 'absolute' percentage change from the normalised baseline control period, in order that the dose-dependent change in

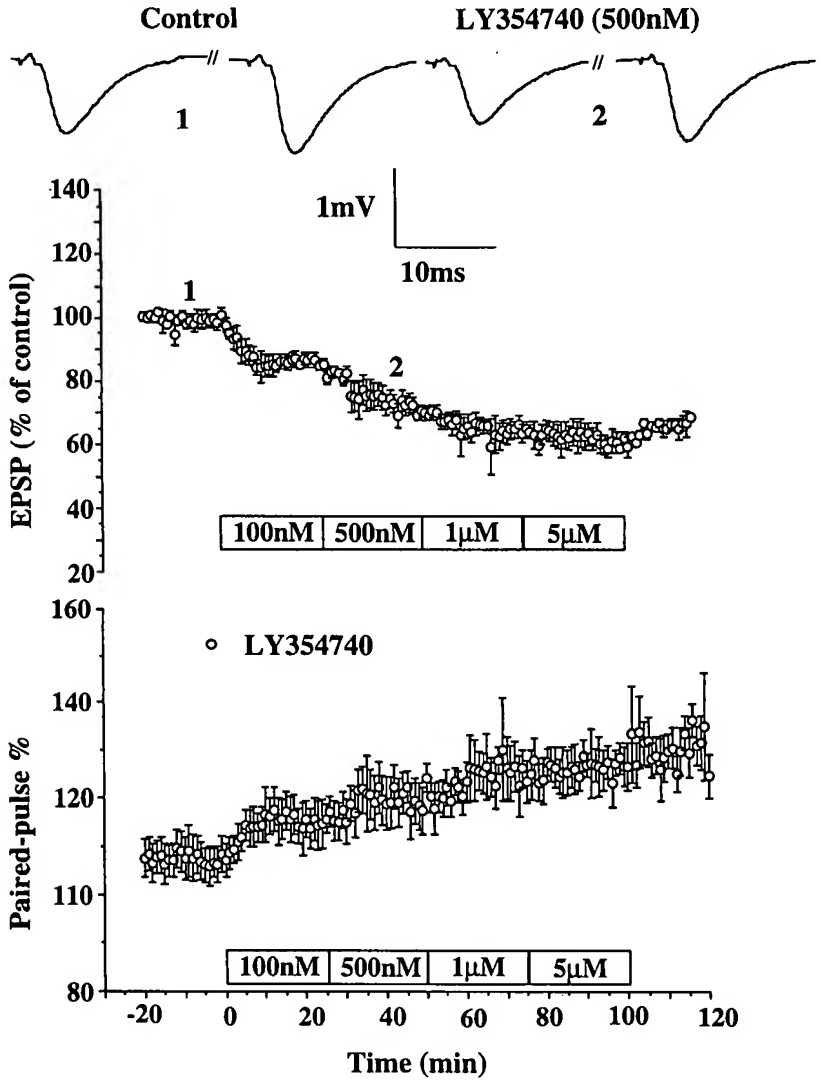


Fig. 3. LY354740 causes a dose-dependent inhibition of excitatory synaptic transmission in the lateral perforant path of the dentate gyrus in vitro. Upper graph, subsequent to a 20 min baseline period, perfusion of LY354740 at doses of 100 nM, 500 nM, 1 and 5 μM caused an increasing inhibition in the amplitude of the field EPSPs. Lower graph, paired-pulse facilitation in the lateral perforant path undergoes a concomitant increase in response to increasing doses of LY354740. The original traces show pairs of EPSPs elicited with a 40 ms inter-stimulus interval. Note the paired-pulse facilitation under control conditions, indicative of the lateral perforant pathway, and the subsequent increase in paired-pulse facilitation induced by LY354740 (500 nM).

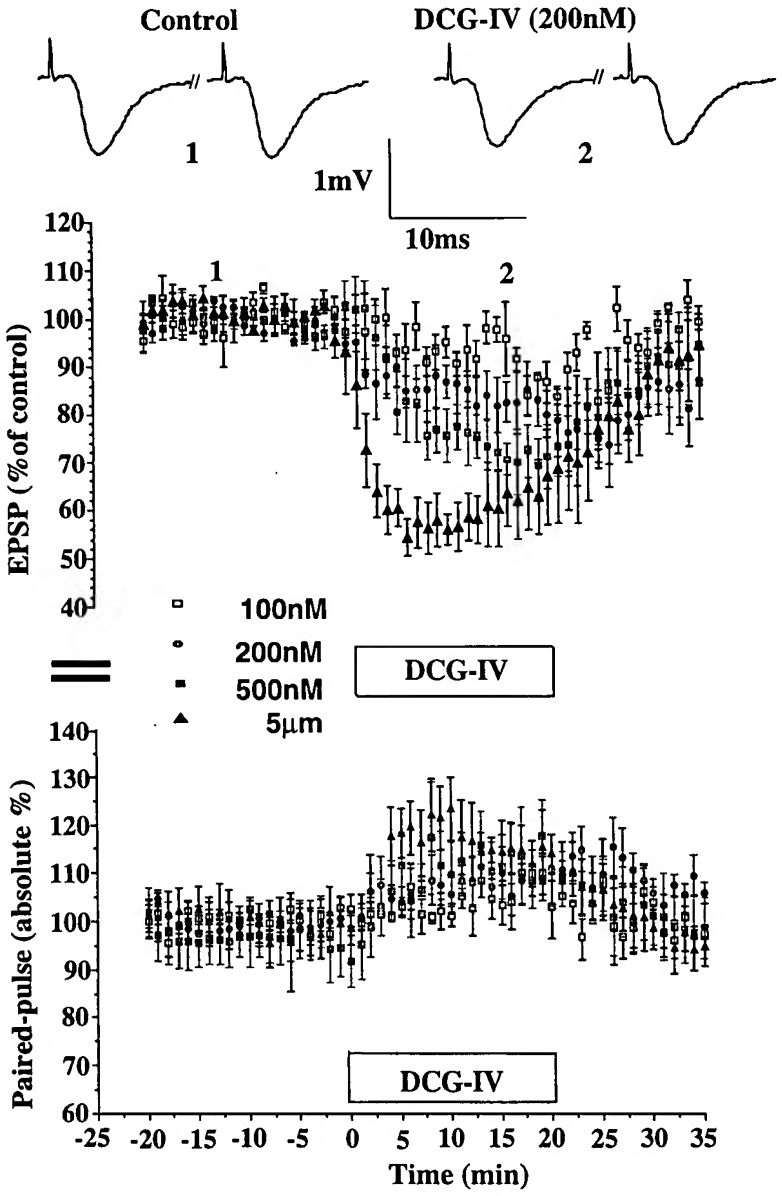


Fig. 4. DCG-IV causes a dose-dependent inhibition of excitatory synaptic transmission in the lateral perforant pathway of the dentate gyrus in vitro. Upper graph, following a stable baseline for 20 min, perfusion of DCG-IV at doses of 100, 200 and 500 nM and 5 μM caused a dose-dependent reduction in the EPSP amplitude. Lower graph, The absolute percentage changes in the level of paired-pulse facilitation induced by the application of stated doses of DCG-IV. The traces show the increase in paired-pulse facilitation in response to the application of 200 nM.

paired-pulse could be easily visualised in the figures with four different doses displayed (compare with Figs. 3 and 5).

All experiments in the dentate gyrus were carried out in the presence of 100 μM picrotoxin to block $GABA_A$ receptor-mediated inhibition. Drugs were added directly to the perfusate after establishing a steady baseline. Each slice was exposed to only one concentration of either LY354740 or DCG-IV except in some of the experiments carried out on LY354740 in the lateral perforant pathway in which cumulative doses were tested. LY354740 was a generous gift from Eli Lilly, USA. DCG-IV was obtained from Tocris Cookson.

Summarised results are expressed as normalised EPSP mean amplitude ± S.E.M. Data was analysed using Student's paired *t*-test, and repeated measures analysis of variance. Dose–response curves were constructed using Graphpad (Prism) software.

3. Results

3.1. LY354740 and DCG-IV inhibit the EPSP in the medial perforant path

The placement of the recording and stimulating electrodes in the medial perforant path was verified by the

presence of paired-pulse depression of EPSPs in response to paired-pulse stimulation.

After establishing a stable amplitude test EPSP for at least 20 min, bath application of LY354740 (20–25 min) caused a dose-dependent and reversible inhibition of the EPSP amplitude in the medial perforant path. The approximate threshold concentration of LY354740 was 10 nM, which induced a small inhibition of the EPSP amplitude of $11 \pm 4\%$ ($P < 0.05$, $n = 5$). LY354740 at higher concentrations of 100 nM and 300 nM reduced the EPSP amplitude by $36\% \pm 2\%$ ($P < 0.05$, $n = 7$) and $62 \pm 6\%$ ($P < 0.05$, $n = 4$), respectively (Fig. 1). Maximal inhibition of $85 \pm 3\%$ ($P < 0.05$, $n = 4$) was evoked with 5 μM LY354740. The dose–response curve for LY354740 (Fig. 6) was best fitted with a one-site binding hyperbola. The EC_{50} was estimated to be 115 ± 16 nM from this dose–response curve.

Evidence that the LY354740-evoked inhibition of the EPSPs was presynaptic was shown by an accompanying reduction in paired-pulse depression. In control media, EPSPs evoked in pairs, at an interval of 40 ms, resulted in paired-pulse depression of about 16%. Paired-pulse depression was reduced by LY354740 in a dose-dependent manner. Thus 10 nM, 100 nM, 300 nM and 5 μM caused absolute percentage changes in paired-pulse depression of $8 \pm 4\%$ ($P < 0.05$, $n = 5$), $21 \pm 12\%$ ($P < 0.05$, $n = 4$), $56 \pm 9\%$ ($P < 0.05$, $n = 4$) and $78 \pm 11.7\%$ ($P < 0.05$, $n = 5$), respectively (Fig. 1).

Perfusion of DCG-IV also resulted in dose-dependent and reversible inhibition of the EPSP amplitude, although less potently than LY354740. In the presence of 100 nM, 200 nM, 500 nM and 5μM DCG-IV, the EPSP amplitude was reduced by $21 \pm 4\%$ ($P < 0.05$, $n = 6$), $36 \pm 2\%$ ($P < 0.05$, $n = 8$), $47 \pm 3\%$ ($P < 0.05$, $n = 7$) and $82 \pm 4\%$ ($P < 0.05$, $n = 4$) of control values, respectively (Fig. 2, also see Fig. 4). The EC_{50} value was estimated to be 317 ± 54 nM from the dose–response curve of Fig. 6 fitted with a one-site binding hyperbola. Similar to that observed with LY354740, the inhibition of the test EPSP by DCG-IV was accompanied by a reduction in paired-pulse depression. For example, in 100 nM, 200 nM, 500 nM and 5 μM, the absolute percentage changes in paired-pulse were

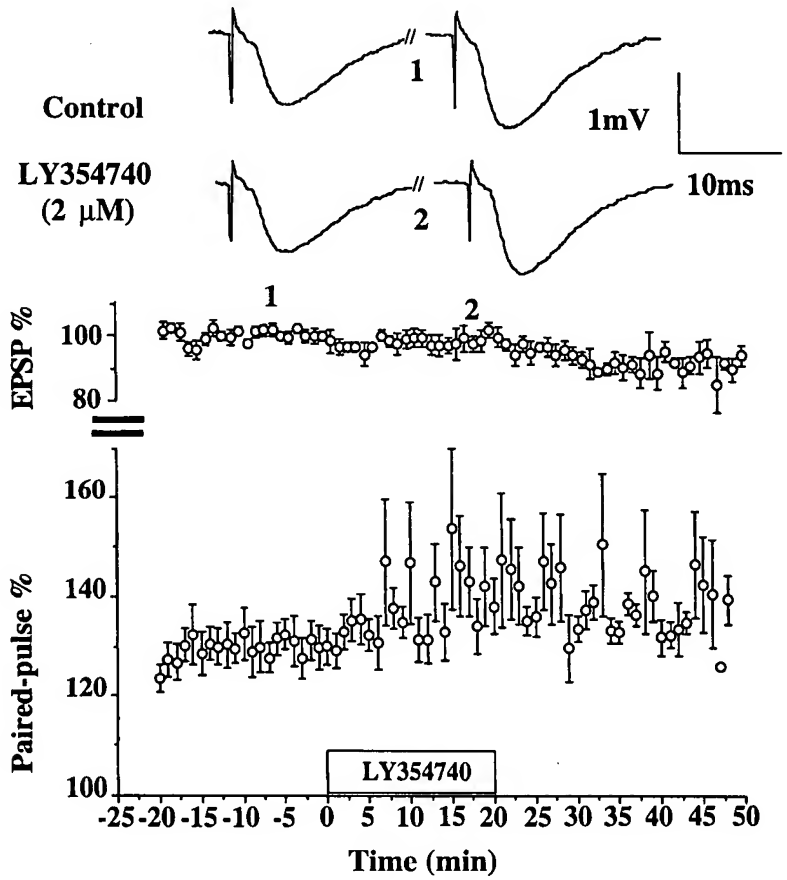


Fig. 5. Dose–response curves for the action of LY354740 and DCG-IV in the medial and lateral perforant paths. The dose–response curves were best fitted with a one-site binding hyperbola (Prism software) $Y = B_{max} \cdot X/(K_d + X)$ where B_{max} is the maximal binding, and K_d is the concentration of ligand required to reach half-maximal binding. The EC_{50} for LY354740 was estimated to be 115 ± 16 nM and 230 ± 58 nM in the medial and lateral perforant paths, respectively. The EC_{50} for DCG-IV was estimated to be 317 ± 54 nM and 334 ± 69 nM in the medial and lateral perforant paths, respectively. Each point plots the mean ± S.E.M. values for four to eight slices.

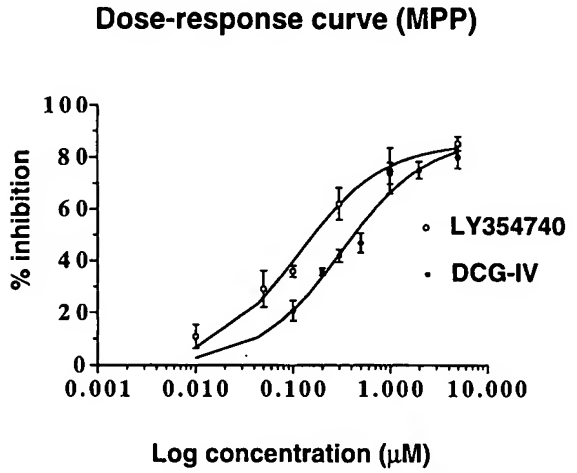
11 ± 3% ($P < 0.05$, $n = 6$), 29 ± 6% ($P < 0.05$, $n = 7$), 42 ± 8% ($P < 0.05$, $n = 7$) and 89 ± 19% ($P < 0.05$, $n = 4$; Fig. 2), respectively.

3.2. LY354740 and DCG-IV inhibit the EPSP in the lateral perforant path

The placement of the recording and stimulating electrodes in the lateral perforant path was verified by the presence of paired-pulse facilitation of EPSPs in response to paired-pulse stimulation.

Both LY354740 and DCG-IV inhibited the EPSP in the lateral perforant path, but with a lower potency and lower maximal inhibition than in the medial perforant path. The EPSP amplitude was significantly reduced by 15 ± 2%, 28 ± 3%, 46 ± 4% and 48 ± 4% in 100 nM, 500 nM, 1 μM and 5 μM LY354740, respectively (Fig. 3, also see Fig. 6). All values were significant ($P < 0.01$, $n = 5$) when tested using a repeated measures analysis of variance. The EC_{50} value was estimated to be 230 ± 58 nM from the dose–response curve (Fig. 6). A dose-dependent significant increase in paired-pulse facilitation accompanied the LY354740-induced depression of the EPSPs, in the lateral perforant path. In control conditions the paired-pulse facilitation was 7 ± 4%. This increased to 15 ± 4%, 20 ± 4%, 25 ± 3% and 25 ± 4% in 100 nM, 500 nM, 1 μM and 5 μM, respectively. All values were significant when tested using the repeated measures analysis of variance ($P < 0.01$, $n = 5$) (Fig. 3).

DCG-IV also inhibited the EPSP in the lateral perforant path, but like the medial perforant path, with a lower potency than LY354740. Perfusion of DCG-IV reduced the EPSP amplitude by 7 ± 3%, 16 ± 5%, 17 ± 5% and 39 ± 7% in 100 nM, 200 nM, 500 nM and 5 μM, respectively. All values were significant when tested using the Student's paired t-test ($P < 0.01$, $n = 5$) (Fig. 4). The estimated EC_{50} value was 334 ± 69 nM. Like that with LY354740, the increase in paired-pulse facilitation was relatively small and was dose-dependent. DCG-IV caused an absolute percentage increase in paired-pulse facilitation of 7 ± 4%, 9 ± 4%, 13 ± 7% and 14 ± 6% in 100 nM, 200 nM, 500 nM and 5 μM, respectively. All values were significant when tested using the Student's paired t-test ($P < 0.01$, $n = 5$) (Fig. 4).



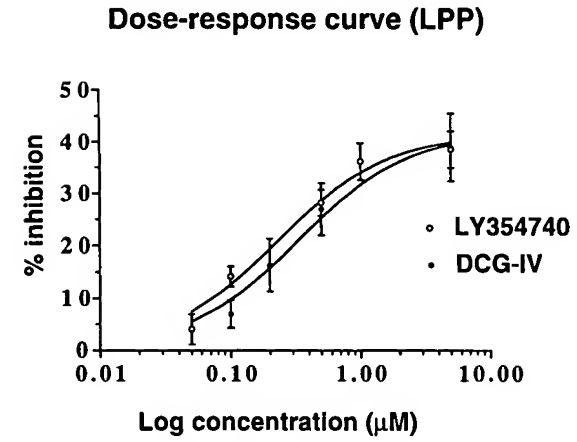


Fig. 6. LY354740 does not produce an inhibition of excitatory synaptic transmission in the CA1 hippocampus. Upper graph, application of 2 μM LY354740 does not produce an inhibition of the EPSP in CA1. Lower graph, LY354740 does not produce a significant change in paired-pulse facilitation in CA1.

3.3. LY354740 has no effect on EPSP amplitude in CA1

LY354740 was applied at a concentration which was sufficient to maximally depress synaptic transmission in both pathways of the dentate gyrus (2 μM). At this concentration LY354740 had no discernible effect, 98.9 ± 2.6% ($P > 0.05$, $n = 4$; Fig. 5).

4. Discussion

The present electrophysiological study has shown that LY354740 is a potent agonist at the group II mGlu receptor responsible for mediating inhibition of EPSPs at the medial perforant path in the dentate gyrus in vitro, with the threshold dose about 10 nM and the EC_{50} close to 100 nM. This is the most potent agonist action at group II mGlu receptors, demonstrated in electrophysiological studies, in this pathway. In comparison with other agonists, electrophysiological studies have shown that DCG-IV has an EC_{50} of close to 300 nM (present study), while LCCG-1 has an EC_{50} of 30 μM (Ugolini and Bordi, 1995). The particularly potent properties of LY354740 are in agreement with neurochemical studies in which LY354740 has been shown to be the most potent group II agonist synthesised, with an EC_{50} of 5 nM and 24 nM for the inhibition of forskolin-stimulated cAMP at expressed mGluR2 receptor and mGlu receptors, respectively (Schoepp et al., 1997b)

and a similar potency for group II mGlu receptor in the rat cerebral cortex and hippocampus group II mGlu receptor (Monn et al., 1997; Schoepp et al., 1997b).

Both LY354740 and DCG-IV were also found to cause a depression of the EPSP in the lateral perforant path, although this path was less sensitive to the agonists, the EC_{50} for LY354740 and DCG-IV being close to 200 nM and 300 nM, respectively, much higher than in the medial path. In addition, the maximal inhibition (40–50%) was much lower than in the medial path (80–90%). Such a reduced sensitivity in the lateral perforant path is likely to reflect a lower density of group II mGlu receptors in this path. A difference in the extent of inhibition by group II mGlu receptor agonists between the medial and lateral perforant path was shown previously by Macek et al. (1996), although this study also found a much lower sensitivity to DCG-IV in both the medial and lateral perforant path than the present study, with the EC_{50} for the action of DCG-IV being 1.6 μM and >3 μM, respectively. This lower sensitivity in the study of Macek et al. (1996) may be due to a developmental decrease in group II mGlu receptor sensitivity, similar to that occurring in CA1 (Vignes et al., 1995; Shigemoto et al., 1997). Thus Macek et al. (1996) used animals of 100–150 g weight, compared to 50–70 g weight in the present study. However, we have not found any developmental decrease in sensitivity to group II mGlu receptor agonists in the medial perforant path of the dentate gyrus (unpublished results). The complete lack of effect of LY354740 in the CA1 region of the hippocampus reflects the absence of group II mGlu receptors in this region of the adult rat, and confirms previous electrophysiological studies using lower potency agonists than LY354740, such as DCG-IV (Gereau and Conn, 1995; Breakwell et al., 1997)

The inhibition of the EPSP by LY354740 and DCG-IV was accompanied by a change in paired-pulse depression/facilitation. Such changes demonstrate that the group II mGlu receptor agonists caused a decrease in the presynaptic probability of transmitter release. The most likely receptor mediating the presynaptic inhibition demonstrated in the present study is mGluR2. Several immunohistochemical studies have shown that mGluR2 is located at a very high density on perforant path axons in the molecular layer of the dentate gyrus, especially in the medial perforant path (Ohishi et al., 1993; Neki et al., 1996; Petralia et al., 1996; Shigemoto et al., 1997). We cannot rule out inhibition via mGluR3 receptors, although this receptor is only present at a much lower density in the hippocampus and is mainly located on glial cells (Petralia et al., 1996). Activation of the group II mGlu receptors may inhibit Ca^{2+} influx, thereby reducing the probability of transmitter release. Alternatively, as the mGluR2 receptors are located preterminally at some distance from the transmitter release sites and corresponding Ca^{2+} channels, rather than presynaptically (Shigemoto et al., 1997), activation of mGluR2 receptors may be linked to opening of K^+ channels, a reduction in the amplitude/duration of the axonal action potential, and thereby reduction of Ca^{2+} influx and the probability of transmitter release.

Acknowledgements

This work was supported by grants from the Health Research Board, Ireland, the Wellcome Trust and the European Union.

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Pergamon

0197-0186(93)E0023-9

Neurochem. Int. Vol. 24, No. 5, pp. 439–449, 1994

0197-0186/94 $7.00+0.00

COMMENTARY

NOVEL FUNCTIONS FOR SUBTYPES OF METABOTROPIC GLUTAMATE RECEPTORS

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(Received 18 October 1993; accepted 9 December 1993)

Abstract—Metabotropic or "G-protein coupled" glutamate receptors (mGluRs) were discovered and established as a new type of excitatory amino acid receptor by their unique coupling mechanism (phosphoinositide hydrolysis) and pharmacological characteristics. Recently, the cloning of mGluRs and the availability of selective pharmacological agents has greatly increased knowledge of these receptors. It is now recognized that mGluRs are a highly heterogenous family of glutamate receptors with novel molecular structure that are linked to multiple second messenger pathways. Members of this family have unique pharmacological properties and function to modulate the presynaptic release of glutamate and the postsynaptic sensitivity of the cell to glutamate excitation. New information on mGluRs is elucidating the functions of mGluR subtypes in normal and pathological aspects of neuronal transmission. Basic knowledge of the role of specific mGluRs in CNS function and pathologies will further expand in the near future. This knowledge is providing the framework for the discovery of novel pharmacological approaches to modulate excitatory amino acid neuronal transmission.

L-Glutamate is an endogenous excitatory amino acid neurotransmitter substance that contributes excitatory input into the majority of synapses in the central nervous system. Recent molecular studies have demonstrated that glutamate acts on two major classes of receptors, ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs).

iGluRs are ligand-gated integral ion channels that are composed of multiple subunit proteins. Each subunit protein is characterized by a relatively large N-terminal extracellular domain with four hydrophobic membrane spanning regions and selective permeability to monvalent or divalent cations. Based on their primary sequence and their agonist sensitivity when expressed, iGluRs are classified into three types: *N*-methyl-D-aspartate (NMDA), kainate, and AMPA receptors (see Barnard and Henley, 1990; Sommer and Seeburg, 1992; Barnes and Henley, 1992).

mGluRs were initially characterized as G-protein linked (i.e. pertussin toxin sensitive) glutamate receptors that were coupled to activation of phosphoinositide hydrolysis, and thus the mobilization of intracellular calcium (Sugiyama *et al.*, 1987). These receptors were demonstrated to exist in various tissues including cultured neurons (Sladeczek *et al.*, 1985), brain slices (Nicoletti *et al.*, 1986), glia (Pearce *et al.*, 1986), and retinal cells (Osborne, 1990) by the ability of various non-selective mGluR agonists (i.e. quisqualate, ibotenate) to increase phosphoinositide hydrolysis. Later it was found that the rigid glutamate analog (±)*trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*trans*-ACPD) (Palmer *et al.*, 1989; Desai and Conn, 1990) or its 1S,3R- isomer (1S,3R-ACPD) (Irving *et al.*, 1990; Schoepp *et al.*, 1991) activates phosphoinositide hydrolysis in CNS tissues at concentrations with no effect on iGluRs. The availability of this first selective mGluR agonist allowed investigators to begin probing the cellular consequences of selectively activating mGluRs (see Schoepp and Conn, 1993).

It is now known that 1S,3R-ACPD acts on multiple mGluRs, and this leads to a variety of biochemical responses in the target cell (see Table 1; Schoepp and Conn, 1993). mGluR activation with 1S,3R-ACPD has also been shown to modulate the excitability of a number of cell types through a variety of mechanisms (see Table 1). For example, 1S,3R-ACPD inhibits slow afterhyperpolarization (I_{AHP}) that is responsible for accomodation, and this results in repetitive firing of these neurons (Stratton *et al.*, 1989; Charpak and

Table 1. Biochemical and cellular consequences of *in situ* mGluR activation[a]

(1) Changes in second messenger systems	
↑ Phospholipase C	↑ Phospholipase D
↓ cAMP	↑ cAMP
↑ Arachidonic acid	↑ cGMP
(2) Modulation of ion channel currents (1)	
↓ I_{AHP}	↓ I_M
↓ I_K (Ca^{2+}-independent)	↑ I_K(CA^{2+}-dependent)
↓ $I_{Ca^{2+}}$ (*N*-type)	↑ $I_{Ca^{2+}}$-(L-type)
(3) Modulation of ligand gating ionotropic receptors	
↑ I_{NMDA}	↑ I_{AMPA}
↓ $I_{Muscimol}$	
(4) Modulation of neurotransmitter release	
↓ Glutamate	↑ Glutamate (PI linked)
↓ GABA	↑ Dopamine
(5) Modulation of synaptic plasticity	
↑ LTP	↑ LTD

[a] Also see Conn and Desai (1991) and Schoepp and Conn (1993).

Gahwiler, 1991). 1S,3R-ACPD-sensitive mGluRs also mediate inhibition of voltage sensitive calcium channels in some neurons (Swartz and Bean, 1992; Trombley and Westbrook, 1992; Sayer *et al.*, 1992), facilitate of iGluR-mediated currents (Aniksztejn *et al.*, 1992; Kelso *et al.*, 1992; Collins, 1993; Bleakman *et al.*, 1992), and participate in long-term changes in neuronal excitability such as long-term-potentiation (LTP) (Ito and Sugiyama, 1991; Aniksztejn *et al.*, 1992; Zheng and Gallagher, 1992; Izumi *et al.*, 1991; Behnisch and Reyman, 1993; Bashir *et al.*, 1993) and long-term-depression (LTD) (Linden *et al.*, 1991; Liu *et al.*, 1993). However, an association between these functional effects of 1S,3R-ACPD and the activation of phosphoinositide hydrolysis or alterations of cAMP formation have been difficult to establish (see Schoepp and Conn, 1993). It is now known that mGluR subtypes fall into three groups based on their pharmacological characteristics and second messenger coupling (see Table 2, and text below). With the knowledge gained by the cloning and characterization of mGluR subtypes, the molecular basis for these effects of 1S,3R-ACPD and other mGluR agonists are now being revealed. This article focuses on select aspects of recent structural and pharmacological information which has enhanced understanding of the cellular mechanisms associated with activation of mGluR subtypes. More comprehensive reviews on mGluR molecular structure and the cellular consequences of mGluR activation have been published elsewhere (see Schoepp *et al.*, 1990; Conn and Desai, 1991; Baskys, 1992; Barnes and Henley, 1992; Mayer and Miller, 1990; Miller, 1991; Nakanishi, 1992; Schoepp and Conn, 1993).

CLONING AND CHARACTERIZATION OF A HETEROGENOUS FAMILY OF mGluRs

The *xenopus* oocyte offers a sensitive system which can be used to express and study phosphoinositide coupled membrane receptors. Early work showed that mGluRs could be expressed following rat brain mRNA injection in the *xenopus* oocyte by their coupling through a pertussis toxin sensitive G-protein to phospholipase C (Sugiyama *et al.*, 1987, 1989). Inositol trisphosphate, which is formed following mGluR activation of phospholipase C, mobilizes intracellular

Table 2. Pharmacology of cloned mGluRs[a]

Receptor	Second messenger	Pharmacological characteristics
Group 1[b]		
mGluR1α	↑ Phosphoinositide hydrolysis	Potently activated by quisqualate and 1S,3R-ACPD
mGluR1β		Insensitive to L-AP4
mGluR1c	Postsynaptic	
mGluR5		
mGluR5b		
Group 2[c]		
mGluR2	↓ cAMP	Potently activated by L-CCG-I and 1S,3R-ACPD
mGluR3	Presynaptic	Insensitive to quisqualate and L-AP4
		Sensitive to pertussis toxin (PTX) inhibition
Group 3[d]		
mGluR4	↓ cAMP	Potently activated by L-AP4 and L-SOP
mGluR6		Insensitive to quisqualate and 1S,3R-ACPD
mGluR7		Sensitive to pertussis toxin (PTX) inhibition

[a] See Nakanishi (1992). [b] Primary references: mGluR1α, Masu *et al.* (1991), Houamed *et al.* (1991); mGluR1β, Tanabe *et al.* (1992); mGluR1c, Pin *et al.* (1992); mGluR5, Abe *et al.* (1992); mGluR5b, Minakami *et al.* (1993). [c] Primary references: mGluR2, Tanabe *et al.* (1992); mGluR3, Tanabe *et al.* (1993). [d] Primary references: mGluR4, Tanabe *et al.* (1993); mGluR6, Nakajima *et al.* (1993), mGluR7, Saugstad *et al.* (1993), Okamoto *et al.* (1994).

calcium, and this could be detected in the *xenopus* oocyte in a highly sensitive manner by the measurement of calcium-activated chloride currents. Using this system, two independent groups expression cloned a phosphoinositide coupled mGluR (now termed mGluR1α) from a rat cerebellar library (Masu *et al.*, 1991; Houamed *et al.*, 1991). The molecular structure of mGluR1α makes it a highly unique G-protein coupled receptor. Like other G-protein coupled receptors that have been cloned, mGluR1α possesses eight hydrophobic regions that likely correspond to a N-terminus signal peptide and seven membrane spanning motifs. However, the primary sequence and size of mGluR1α clearly distinguish it from other G-protein linked receptors. Rat mGluR1α has no significant sequence homology with non-glutamate G-protein-coupled receptors that have been cloned. In addition, mGluR1α is quite large by comparison, with a total size of 1199 amino acids and a molecular size of 133,229 daltons. The size of mGluR1α is remarkable considering that it functions to bind and transduce the effects of a small molecule such as glutamate.

The expression cloning of mGluR1α has rapidly lead to the discovery of a heterogenous family of mGluRs that appear to be coupled to multiple effectors *in situ*. At least seven different mGluR subtypes (mGluR1–mGluR7), along with various alternate splice versions of mGluR1 and mGluR5 (see Table 2), have been cloned. These mGluRs fall into three groups based on their degree of sequence homology and pharmacology. Members within each group have about 70% homology with each other (as opposed to 40% homology between groups). The higher homology within each mGluR group is associated with the expression of the same transduction mechanism and similar agonist pharmacology. These groups include: (1) mGluR1 (α, β, and c forms) and mGluR5 (a and b forms) which are coupled to increased phosphoinositide hydrolysis and are potently activated by quisqualate and with lower potency, *trans*-ACPD; (2) mGluR2 and mGluR3, which are negatively linked to adenylyl cyclase, are most potently activated by *trans*-ACPD, but are insensitive to quisqualate; and (3) mGluR4, mGluR6, and mGluR7, which are negatively coupled to adenylyl cyclase, are potently activated by L-2-amino-4-phosphonobutyrate (L-AP4) and L-serine-O-phosphate (L-SOP), but are relatively insensitive to *trans*-ACPD and quisqualate. Each of these mGluRs has a unique distribution in the CNS and retina, and this likely reflects a diversity of functions in normal and pathological processes (see Nakanishi, 1992).

With the cloning of mGluRs, the functional domains of mGluRs that are responsible for ligand binding and G-protein interaction can now be explored. mGluRs differ from other G-protein coupled receptors by their large size, including the presence of a large N-terminus extracellular domain, and they have no significant sequence homology with other G-protein coupled receptors, even in the transmembrane regions of the receptor. The unique molecular structure of mGluRs thus renders structural models of other G-protein receptors non-applicable, and suggests that the functional domains for mGluRs that are responsible for ligand binding and G-protein interaction may be distinct. The ligand binding domain for other G-protein coupled receptors (i.e. dopamine, serotonin, muscarinic) has been suggested to be in the transmembrane region (see Findlay and Eliopoulos, 1990; Hibert *et al.*, 1991). This does not appear to be the case for mGluRs. A recent study (O'Hara *et al.*, 1993) suggests that the mGluR ligand binding domain may be in the amino-terminus extracellular domain of the protein. In their study they showed that the mGluR extracellular domain is similar in structure to bacterial periplasmic amino acid binding proteins. The mGluR ligand binding site was predicted to be present in the extracellular amino-terminus domain based on regions of similarity between these proteins. Specifically, the side chains of mGluR1 amino acids serine-165 and threonine-188 were hypothesized to hydrogen bind to the α-amino acid backbone of the ligand (glutamate). In further support of this hypothesis, mutants of mGluR1 with non-polar amino acid substitutions at these positions (alanine-165 and/or alanine-188) were shown to reduce agonist (quisqualate and glutamate) potency in activating phosphoinositide hydrolysis and affinity in displacing ^{3}H-glutamate binding.

Consistent with the N-terminus region possessing the glutamate recognition site for mGluRs, it has recently been reported that the pharmacological characteristics of an mGluR subtype, but not its G-protein/second messenger specificity, can be altered by the amino acid composition of the extracellular domain. In the study of Takahashi *et al.* (1993) chimeric mGluRs were prepared, exchanging N-terminus regions of mGluR1α (a quisqualate-sensitive, phosphoinositide linked mGluR), with sequences from mGluR2 (a quisqualate-insensitive negatively-coupled cAMP-linked mGluR). Such chimerics retained mGluR1α-like coupling to phosphoinositide hydrolysis, as measured by glutamate-induced currents in the *xenopus* oocytes. However, the chimeric mGluR1α receptors lost their wild-type quisqualate

sensitivity, and gained sensitivity to the agonists *trans*-ACPD and (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), which are more potent at wild type mGluR2 receptors compared to mGluR1α. These data clearly show that the pharmacological differences between mGluR subtypes are confered in the amino acid composition of the extracellular region of the mGluR. Information such as this may be useful to create 3-D protein structural models of the mGluR ligand recognition sites. Such molecular models might be used to design highly specific ligands for mGluR subtypes in the future.

CELLULAR FUNCTIONS OF PHOSPHOINOSITIDE LINKED mGluRs

The phosphoinositide coupled mGluRs (mGluR1 and mGluR5) are highly sensitive to quisqualate and can be selectively activated by 1S,3R-ACPD (see Table 2). Thus, quisqualate sensitivity at low micromolar concentrations, along with sensitivity to the more selective agonist 1S,3R-ACPD, would be indicative of the specific involvement of mGluR1 and/or mGluR5 subtypes, and possibly the phosphoinositide second messenger system, in mediating a particular *in situ* mGluR response. Consistent with this, both quisqualate and 1S,3R-ACPD have been demonstrated to mobilize intracellular calcium stores in glia (Glaum *et al.*, 1990; Ahmed *et al.*, 1990) and in dendritic processes and the cell soma of neurons (Murphy and Miller, 1988; Irving *et al.*, 1992; Vranesic *et al.*, 1991), likely via inositol-1,4,5-triphosphate induced calcium release. The activation of phosphoinositide linked mGluRs also increases diacylglycerol formation and thus enhances protein kinase C activity (Manzoni *et al.*, 1990).

A variety of cellular responses that exhibit both quisqualate and 1S,3R-ACPD sensitivity, but are insensitive to blockade by iontropic glutamate receptor antagonists, have been reported. These responses have a pharmacology consistent with the selective activation mGluR1 and/or mGluR5. For example quisqualate and *trans*-ACPD mobilize intracellular calcium and thus activate a large conductance calcium-dependent potassium channel in cultured cerebellar granule cells (Fagni *et al.*, 1991). This would reduce the excitability of these cells to other excitatory inputs such as those from ionotropic glutamate receptor activation. Quisqualate and 1S,3R-ACPD-sensitive mGluRs have also been shown to enhance NMDA receptor mediated currents in hippocampal neurons (Aniksztejn *et al.*, 1991) or in the *xenopus* oocyte (Kelso *et al.*, 1992) via the activation of protein kinase C. However, in the study of Harvey and Collingridge (1993) enhancement of NMDA currents by 1S,3R-ACPD was not blocked by inhibitors of protein kinase C or depletion of intracellular calcium by thapsigargin (Harvey and Collingridge, 1993). Regardless of the underlying mechanism(s), mGluR-mediated enhancement of ionotropic glutamate receptor activation may underly long-term potentiation (Aniksztejn *et al.*, 1992; Bashir *et al.*, 1993) or the induction of seizure states or excitoxicity in animals (Sacaan and Schoepp, 1992) (see Fig. 1).

Quisqualate activates phosphoinositide linked mGluRs at presynaptic sites as well, since it will mobilize intracellular calcium in synaptosomes from the rat cerebral cortex (Adamson *et al.*, 1990). Phosphoinositide linked mGluRs appear to modulate the release of glutamate from presynaptic nerve endings. Both quisqualate and 1S,3R-ACPD enhance the calcium-dependent release of glutamate from synaptosomes of the rat cerebral cortex (Herrero *et al.*, 1992a). Activation of mGluRs in synapsosomes is also linked to a rapid and transient increase in diacylglycerol levels and to subsequent activation of protein kinase C (Sanchez-Prieto *et al.*, 1993). The enhanced release of glutamate which is linked to mGluR activation can be mimicked by phorbol esters that activate protein kinase C, and both effects require the presence of low concentrations of arachidonic acid (Herrero *et al.*, 1992a, b). Activation of postsynaptic ionotropic glutamate receptors (NMDA and AMPA) leads to arachidonic acid formation (Dumuis *et al.*, 1988, 1990) and this arachidonic acid might serve as a retrograde second messenger to facilitate mGluR-mediated positive feedback release of transmitter glutamate (see Nicholls, 1992). Since this mGluR-mediated presynpatic positive feedback mechanism would appear to work in concert with postsynaptic events such as NMDA receptor activation, it may have a physiological role in the maintenance of long-term potentiation of synaptic responses or in pathological conditions of enhanced glutamatergic transmission (i.e. seizures and excitotoxicity) (see Fig. 1). A number of different lines of evidence using the mGluR antagonist compound L-2-amino-3-phosphonopropionic acid (L-AP3) support this hypothesis. L-AP3 has been reported to antagonize 1S,3R-ACPD- and quisqualate-induced phosphoinositide hydrolysis in slices of the rat hippocampus or cerebral cortex (see Schoepp *et al.*, 1990) or in mGluR1α expressing cells (Houamed *et al.*, 1991), antagonize the mGluR-mediated enhanced release of glutamate from synaptosomes (Herrero *et al.*, 1992a, b), block late synaptic long-term potentiation in the hippocampus (Behnisch

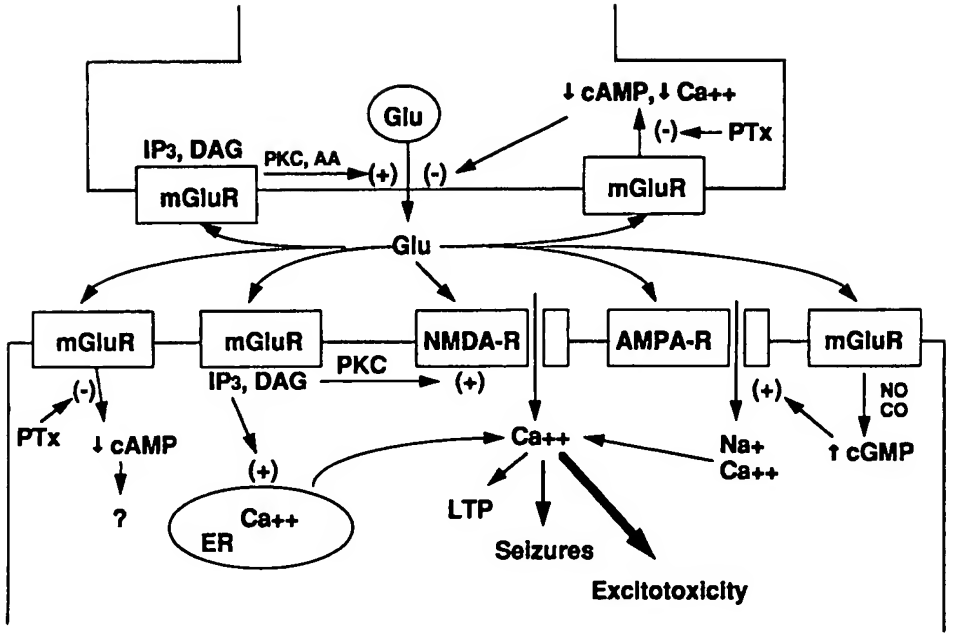

Fig. 1. Mechanisms for mGluR mediated modulation of glutamatergic neuronal transmission. The presynaptic release of glutamate is regulated by both positive and negative feedback mechanisms that involve phosphoionositide-coupled or negatively linked cAMP coupled mGluR autoreceptors, respectively. The specific mGluR subtypes that are localized presynaptically and control glutamate release remain to be determined, and other effectors such as modulation of presynaptic calcium channels may be involved. For negative feedback mGluR autoreceptors, both L-AP4-sensitive and 1S,3R-ACPD-sensitive/L-AP4 insensitive subtypes have been demonstrated. Positive feedback mGluR autoreceptors are linked to phosphoionositide hydrolysis and transduce this effect by the combined effects of diacylglycerol (DAG) and arachidonic acid (AA) to increase protein kinase C (PKC) activity. Postsynaptic quisqualate and 1S,3R-ACPD sensitive mGluRs have been demonstrated to modulate ionotropic glutamate receptor currents, including NMDA and AMPA receptors. In the case of NMDA receptors this may involve PKC mediated phosphorylation subsequent to activation of phosphoionositide coupled mGluRs. AMPA receptor currents are also enhanced by mGluR activation by a process that involves a diffusable second messenger such as carbon monoxide (CO) which can activate guanyl cyclase and increase cGMP. Through these mechanisms pre- and postsynaptic mGluRs function to modulate the excitability of the postsynaptic neurons which are receptive to transmitter glutamate. These modulatory mechanisms may play a physiological role in plasticity phenomenon such as long-term synaptic potentiation (LTP), and pathological states that involve excessive or inappropriate glutamatergic neuronal transmission such as seizure states and the expression of glutamate excitotoxicity.

et al., 1991), block limbic seizures induced by intracerebral administration of 1S,3R-ACPD (Tizzano *et al.*, 1993), and block audiogenic seizures in DBA/2 mice (Klitgaard and Jackson, 1993). Thus, more potent and systemically active antagonist agents which act at the quisqualate- and 1S,3R-ACPD-sensitive mGluR subtypes may offer a novel therapeutic approach to treat epilepsy and various neurodegenerative conditions involving enhanced glutamate transmission and glutamate excitotoxicity.

CELLULAR FUNCTIONS OF cAMP LINKED mGluRs

The discovery and characterization of multiple mGluRs has rapidly expanded understanding about the functions of glutamate receptors in the brain. For example, the second of these to be cloned, mGluR2, was described as a novel mGluR with 46% sequence homology to mGluR1α. Interestingly, mGluR2 was found to be negatively coupled to cAMP formation when expressed in non-neuronal cells (Tanabe *et al.*, 1992). Agonists including the selective mGluR compound *trans*-ACPD and glutamate potently inhibited forskolin-stimulated cAMP formation in CHO cells expressing mGluR2. This effect of these agonists was highly sensitive to inhibition by pertussis toxin. The discovery and characterization of mGluR2 in this manner provided the first convincing evidence that glutamate receptors which are directly linked to

adenylate cyclase via G-proteins may exist *in situ*. In support of this, inhibition of forskolin-stimulated cAMP formation by *trans*- or 1S,3R-ACPD has been demonstrated in neuronal tissues such as brain slices and cultured neurons (Schoepp *et al.*, 1992; Cartmell *et al.*, 1992; Prezeau *et al.*, 1992). Thus, it was recognized that the cellular consequences following mGluR activation with 1S,3R-ACPD could be a result of altered cAMP levels, rather than being linked to the more established *in situ* changes in phosphoinositide hydrolysis.

The availability of cloned mGluRs has facilitated the discovery of more selective pharmacological tools to further facilitate understanding of the specific cellular functions of *in situ* mGluR subtypes. For example, the striatum is a region of the brain which is enriched in glutamatergic afferents arising from cortical–striatal neuronal pathway. In electrophysiological studies using striatal neurons, the mGluR agonist *trans*-ACPD, which acts on multiple mGluRs (see Table 2), depresses synaptic transmission presumably by acting on a presynaptic site to decrease glutamate release (Lovinger, 1991). In subsequent work it was reported that this effect of *trans*-ACPD was not mimicked by L-AP4 (Lovinger *et al.*, 1993). Thus, a *trans*-ACPD-sensitive but L-AP4 insensitive mGluR subtype (such as mGluR2) appears responsible for this presynaptic mGluR effect. Using mGluR2 expressing non-neuronal cells it has been demonstrated that mGluR2 receptors can be selectively activated with (1S,3S,4S)-(carboxycyclopropyl)glycine (L-CCG-I) ($EC_{50} = 0.3$ μM) (Hayashi *et al.*, 1992). L-CCG-I has much less affinity for quisqualate-sensitive phosphoinositide linked mGluRs, or L-AP4 sensitive cAMP linked mGluRs (Hayashi *et al.*, 1992). Thus, L-CCG-I appears useful to probe the cellular functions of mGluR2 activation *in situ*. Consistent with a role for mGluR2 in the regulation of glutamate release in the striatum, L-CCG-I inhibits the depolarization-evoked release of ^{3}H-D-aspartate from slices of the rat striatum (Lombardi *et al.*, 1993). Inhibition of release was demonstrated at concentrations of L-CCG-I that inhibit forskolin-stimulated cAMP formation, but have no effects on phosphoinositide hydrolysis. Consistent with this, other negatively coupled cAMP receptors (i.e. α-adrenergic) have also been linked to modulation of exocytosis. However, it should be noted that the effect of these receptors on exocytosis is not necessarily mediated by their effects on cyclic AMP formation (see Limbird, 1988). Thus, glutamate autoreceptors that are negatively coupled to cAMP formation may be present on glutamatergic nerve terminals in the rat striatum. These mGluRs may act by this transduction mechanism or another (i.e. inhibition of calcium channels) to inhibit glutamate release. The availability of specific mGluR antibodies could ultimately be used to verify a presynaptic localization of specific mGluR proteins in different areas of the brain. The presence of 1S,3R-ACPD sensitive/L-AP4 insensitive glutamate receptor that functions to limit the further release of glutamate by an autoreceptor mechanism offers an additional strategy for the discovery of drugs to control pathological conditions of enhanced glutamatergic transmission in selected synapses where these receptors are present (see Fig. 1).

The role of postsynaptic or even glial mGluRs that are negatively linked to cAMP formation remains to be investigated. Presumably, such receptors would modulate cell excitability by decreasing cAMP-dependent protein kinase activity in the cell. Activators of adenylate cyclase such as forskolin enhance glutamate and kainate induced currents in hippocampal neurons (Greengard *et al.*, 1991). Furthermore, inhibition of cAMP-dependent protein kinase activity with competitive inhibitors of this enzyme inhibits kainate induced currents in neurons (Wang *et al.*, 1991). Since kainate receptors are regulated by cAMP-dependent protein kinases and mGluRs control kinase activity, modulatory iGluR/mGluR interactions are plausible. Hypothetically, decreases in cellular cAMP levels induced by activation of postsynaptic receptors such as mGluR2 or mGluR3 could lower kainate induced synaptic currents and thus effectively decrease the excitability of postsynaptic cells where these receptors are co-localized (see Fig. 1). The availability of selective agonists such as L-CCG-I or DCG-IV for the group two negatively coupled cAMP linked mGluRs will allow further exploration of specific biochemical events that underly mGluR/iGluR interactions.

Cyclic AMP coupled mGluRs may also function to modulate neuronal excitability by altering transmission at inhibitory synapses. In the accessory olfactory bulb of the rat the mGluR2 selective agonist DCG-IV reduces GABA-mediated inhibitory postsynaptic currents (Hayashi *et al.*, 1993). mGluR2 is localized to granule cell dendrites in this region (Hayashi *et al.*, 1993) which form dendrodendric synapses with excitatory mitral cells. Thus, this effect of the mGluR2 selective agonist DCG-IV may reflect a mechanism to remove GABA-mediated inhibitory influences on the excitatory mitral cells and enhance olfactory sensory transmission.

L-AP4-sensitive presynaptic glutamate receptors that control glutamate release were initially described

by Koerner and Cotman (1981). They demonstrated that micromolar concentrations of L-AP4 will inhibit perforant path synaptic transmission from the entorhinal cortex to the dentate gyrus in the rat. This initial finding and other observations of L-AP4-induced supression of neuronal transmission in pathways such as the lateral olfactory tract in the rat (Hori *et al.*, 1982; Collins, 1982), spinal cord responses in the rat (Davies and Watkins, 1982), and mossy fiber inputs to CA3 regions of the hippocampus in the guinea pig (Yamamoto *et al.*, 1983; Lanthorn *et al.*, 1984) were the basis for the existence of a somewhat enigmatic "AP4" glutamate receptor subtype (see Monaghan *et al.*, 1989). For a number of years the cellular/molecular basis for classifying this receptor as either "ionotropic" or "metabotropic" was not clear. Likewise, L-AP4 sensitive receptors have been characterized in the retina (Slaughter and Miller, 1981), where this compound mimics the effects of glutamate by hyperpolarizing the ON-bipolar cells (Slaughter and Miller, 1981; Neal *et al.*, 1981; Miller and Slaughter, 1986). The recent cloning of at least three different L-AP4-sensitive mGluRs (see Table 2) has now indicated that "L-AP4" sensitive glutamate receptors are G-protein linked "metabotropic" receptors. Of the L-AP4-sensitive mGluRs, the mGluR4 receptor is a candidate for a presynaptic autoreceptor that functions at perforant path synapses to decrease glutamate release. Message for mGluR4 is highly expressed in the entorhinal cortex (Thomsen *et al.*, 1992) and L-AP4 and L-serine-O-phosphate (L-SOP), two compounds which suppress synaptic transmission in this pathway (Ganong and Cotman, 1982), are potent agonists in inhibiting cAMP formation in mGluR4 expressing non-neuronal cells (Kristensen *et al.*, 1993; Thomsen *et al.*, 1992; Tanabe *et al.*, 1993).

OTHER mGluR SECOND MESSENGER SYSTEMS

Interestingly, the expression of mGluR6 is restricted to the retina (Nakajima *et al.*, 1993). When expressed in CHO cells, mGluR6 (like mGluR4 and mGluR7) can be potently activated by L-AP4 and L-SOP. For mGluR4, mGluR6, and mGluR7 this is detected by inhibition of forskolin-stimulated cAMP formation. However, L-AP4 receptors in the retina which function to hyperpolarize ON-bipolar cells might do so by stimulation of cGMP phosphodiesterase in a G-protein dependent manner (see Nawy and Jahr, 1990, 1991; Shiells and Falk, 1992). It is possible L-AP4-sensitive retinal receptors such as mGluR6 are coupled to cGMP phosphodiesterase in its *in situ* environment (see Nakajima *et al.*, 1993). Thus, negative coupling of mGluR6 to adenylate cyclase in the CHO cell may be an example of non-physiological coupling in the absence of the native *in situ* effector for the receptor.

Activation of *in situ* mGluRs in some tissues also suggest the direct or indirect coupling to other novel second messengers. In the nucleus tractus solitarius (NTS) of the rat, 1S,3R-ACPD enhances AMPA-induced currents and suppresses $GABA_A$ currents induced by muscimol. These mGluR effects of 1S,3R-ACPD are not linked to alteration in adenylate cyclase or phospholipase C, but appear to involve the activation of soluble guanylate cyclase (Glaum and Miller, 1993a). Interestingly, the mGluR mediated activation of guanylate cyclase in this system involves the novel diffusable second messenger carbon monoxide. 1S,3R-ACPD-induced responses in the NTS are blocked by Zn-protoporphyrin-IX, a heme oxygenase inhibitor that would inhibit the production of carbon monoxide (Glaum and Miller, 1993b). Nitric oxide can also activate guanylate cyclase, but it does not appear to be involved in the NTS response to 1S,3R-ACPD since the nitric oxide synthase inhibitor L-ω-nitroarginine had no effect. However, in rat cerebellar slices 1S,3R-ACPD increases cGMP levels through a nitric oxide dependent mechanism (Okada, 1992).

A single mGluR receptor protein can also cross-talk with multiple second messengers in the same cell. For example, when rat mGluR1α was expressed in CHO cells it was shown that quisqualate activation lead to enhanced phosphoionositide hydrolysis, enhanced cAMP formation, and increased arachidonic acid release (Aramori and Nakanishi, 1992). The coupling of a single mGluR to these multiple effectors (phospholipase C, adenylate cyclase, and phospholipase A_2, respectively) in the same cell appears to involve different G-proteins, since each response was differentially affected by pertussis toxin. Interestingly, when mGluR1α was expressed in another cell (baby hamster kidney, BHK) activation by quisqualate enhanced phosphoinositide hydrolysis but did not appreciably increase cAMP formation (Thomsen *et al.*, 1993). Thus, the *in situ* environment, possibly the stoichiometry of mGluRs, G-proteins, and their effectors in a particular cells type may be an important factor in determining ultimate mGluR cellular functions.

In situ mGluRs activation has also been associated with the activation of phospholipase D (Boss and Conn, 1992; Holler *et al.*, 1993), enhanced cAMP formation through the potentiation of other receptors (i.e. adenosine) that are directly coupled to adenylyl

cyclase via G_s (Casabona *et al.*, 1992; Winder and Conn, 1992, 1993; Alexander *et al.*, 1992), and possibly the direct coupling to ion (i.e. Ca^{2+}) channels (Lester and Jahr, 1990). The specific mGluRs involved in these responses and effects of second messengers linked to these mechanisms on neuronal excitability also remain to be investigated.

SUMMARY

Recent molecular biological information about the structure and pharmacological characteristics of mGluRs subtypes has begun to enhance understanding of cellular mechanisms linked to their activation. It is now apparent that a myriad of pre- and postsynaptic mechanisms exist by which *in situ* expressed mGluRs could modulate cell function in the CNS, and the role of specific mGluR subtypes can now be investigated. More selective agonist and antagonist compounds than those currently known will be needed to sort out the seemingly complex cellular functions of mGluR subtypes. Such agents offer novel approaches to modulate glutamatergic neuronal transmission in very select areas of the CNS.

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Pergamon

0028-3908(95)00093-3

Neuropharmacology Vol. 34, No. 8, pp. 973–982, 1995

0028-3908/95 $9.50 + 0.00

Pharmacological Evidence for an Involvement of Group II and Group III mGluRs in the Presynaptic Regulation of Excitatory Synaptic Responses in the CA1 Region of Rat Hippocampal Slices

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(Accepted 22 May 1995)

Summary—The actions of four mGluR antagonists, (+)-MCPG, MAP4, MCCG and (*S*)-4CPG, were evaluated against agonist-induced depressions of synaptic transmission at the Schaffer collateral–commissural pathway in rat hippocampal slices. (+)-MCPG (1 mM) reversed very effectively depressions of field EPSPs induced by (1*S*,3*R*)-ACPD and (1*S*,3*S*)-ACPD but had weak and variable effects on depressions induced by L-AP4. It had no effect on depressions induced by either (−)-baclofen or carbachol. In contrast, MAP4 (500 μM) reversed very effectively depressions induced by L-AP4 without affecting depressions induced by (1*S*,3*S*)-ACPD. MCCG (1 mM) had the opposite activity; it antagonized depressions induced by (1*S*,3*S*)-ACPD but not those induced by L-AP4. Finally, (*S*)-4CPG (1 mM) reversed small depressions of field EPSPs induced by high concentrations (50–100 μM) of (1*S*,3*R*)- and (1*S*,3*S*)-ACPD, but not L-AP4, whilst having no effect on large depressions induced by 10 μM (1*S*,3*S*)-ACPD in voltage-clamped cells. These results confirm and extend the effectiveness and selectivity of (+)-MCPG as an mGluR antagonist. The divergent effects of the group I antagonist, (*S*)-4CPG, can be explained by an indirect action on postsynaptic receptors which is manifest when high agonist concentrations are used in non-voltage-clamp experiments. The action of MCCG and MAP4 indicates that two pharmacologically-distinct mGluRs, belonging to classes II and III, can regulate synaptic transmission in the CA1 region via presynaptic mechanisms.

Keywords—Metabotropic glutamate receptors (mGluR), ACPD, L-AP4, (+)-MCPG, MCCG, (*S*)-4CPG, MAP4, hippocampus

Activation of metabotropic glutamate receptors (mGluRs) produces multiple effects within the CA1 region of the hippocampus, including a depression of excitatory synaptic transmission (Pin and Duvoisin, 1995). In particular, it has been shown that the mGluR agonists (1*SR*,3*RS*)-1-aminocyclopentane-1,3-dicarboxylate [(1*SR*,3*RS*)-ACPD], its active constituent (1*S*,3*R*)-ACPD (Irving *et al.*, 1990), and (*S*)-2-amino-4-phosphonobutanoate (L-AP4) depress synaptic transmission at Schaffer collateral–commissural synapses via a presynaptic site of action (Baskys and Malenka, 1991; Harvey *et al.*, 1991; Desai *et al.*, 1992). It has also been shown that the mGluR antagonist α-methyl-4-carboxyphenylglycine (MCPG) inhibits the presynaptic depressant actions of (1*S*,3*R*)-ACPD at this synapse (Davies *et al.*, 1993; Manzoni *et al.*, 1994; Bolshakov and Siegelbaum, 1994; Watkins and Collingridge, 1994).

In the present study, we have extended our analysis of the antagonist actions of (+)-MCPG (Davies *et al.*, 1993) on agonist-induced depressions of excitatory synaptic transmission, in the CA1 region of rat hippocampal slices. In addition, we have examined the effects of three antagonists which are selective/specific for the three mGluR subgroups; group I—(*S*)-4-carboxyphenylglycine [(*S*)-4CPG], group II—2*S*,1′*S*,2′*S*-2-methyl-2-(2′-carboxycyclopropyl)glycine (MCCG), and group III—(*S*)-2-methyl-2-amino-4-phosphonobutanoate (MAP4) (Jane *et al.*, 1994; Watkins and Collingridge, 1994).

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METHODS

Experiments were performed on hippocampal slices obtained from Wistar rats (approx 2 weeks old) as described previously (Davies *et al.*, 1990). Coronal slices (400 μm thick) containing hippocampus were cut using a Campden vibroslicer. The hippocampal region was dissected from these slices and area CA3 removed. The slices were stored at room temperature for at least 1 hr before being transferred to either an interface or submerged recording chamber where they were maintained at either 28°–32°C or at room temperature and perfused at a rate of approx 2 ml min^{-1} with medium which comprised (mM): NaCl 124; KCl 3; $NaHCO_3$ 26; $CaCl_2$ 2; $MgSO_4$ 1; D-glucose 10; NaH_2PO_4 1.25, bubbled with a 95% O_2/5% CO_2 mixture. Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) were obtained from *stratum radiatum* using glass microelectrodes (4 MΩ) filled with NaCl (4 M) connected to an Axoclamp-2 amplifier. Whole-cell patch-clamp recordings of excitatory postsynaptic currents (EPSCs) were obtained from *stratum pyramidale* using glass microelectrodes (5–7 MΩ; seal resistances approx 10 GΩ) filled with (mM): $CsMeSO_4$ 130; NaCl 1; $MgCl_2$ 1; $CaCl_2$ 0.035; EGTA 0.05; QX-314 5; HEPES 5 (pH 7.3) connected to an Axopatch-1D amplifier, as described previously (Clark and Collingridge, 1995). Neurones were voltage-clamped at −60 mV. EPSCs were isolated by the addition of picrotoxin (50 μM) to the perfusate and by the inclusion of Cs^+ in the patch pipette. Drugs were administered by addition to the superfusing medium and were applied for a sufficient period to allow their full equilibration.

RESULTS

Agonist actions

Field EPSPs, evoked by low frequency stimulation of the Schaffer collateral–commissural pathway, were depressed by (1*S*,3*R*)-ACPD (25–200 μM; $n = 37$) in a reversible manner. The mean ± SEM depressions were 31 ± 14% ($n = 18$) and 37 ± 12% ($n = 16$) for doses of 50 and 100 μM, respectively. In contrast, the presynaptic fibre volley was unaffected by (1*S*,3*R*)-ACPD. The threshold concentration was approx 25 μM [6 ± 3% depression ($n = 3$)] and the maximum effective concentration approx 100 μM. The depression induced by 100 μM was highly variable between preparations ranging between 9 and 90% but depressions ranging between 20 and 60% were selected to test the antagonists. In the continued presence of (1*S*,3*R*)-ACPD the depression was sustained. Similar sustained but reversible depressions were also induced by (1*S*,3*S*)-ACPD (10–150 μM; $n = 14$), depressions at 50 μM being 41 ± 6% ($n = 8$), and L-AP4 (25–200 μM; $n = 25$), depressions at 50 μM being 41 ± 4% ($n = 3$).

Actions of (+)-MCPG

(+)-MCPG (1 mM) had no effect on field EPSPs *per se* ($n = 3$) but invariably reversed the depressant effects of (1*S*,3*R*)-ACPD (50 μM; $n = 4$). In contrast, (−)-MCPG had no effect on depressions induced by (1*S*,3*R*)-ACPD (50 μM; $n = 2$). A comparison of the actions of the stereo-isomers of MCPG is illustrated in Fig. 1 and pooled data illustrating the

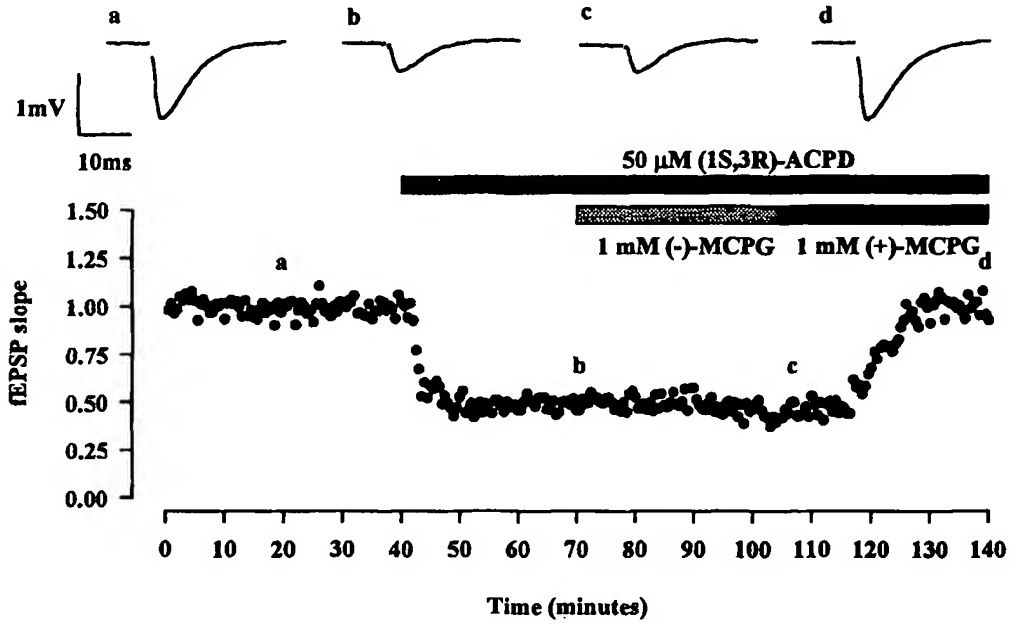

Fig. 1. Stereoselective reversal by MCPG of the depressant action of (1*S*,3*R*)-ACPD. The graph plots the slope of the field EPSP, normalized with respect to the entire illustrated baseline prior to drug administration, vs time. Drugs were applied for the times indicated by bars above the graph and representative traces are shown for the times indicated by a–d.

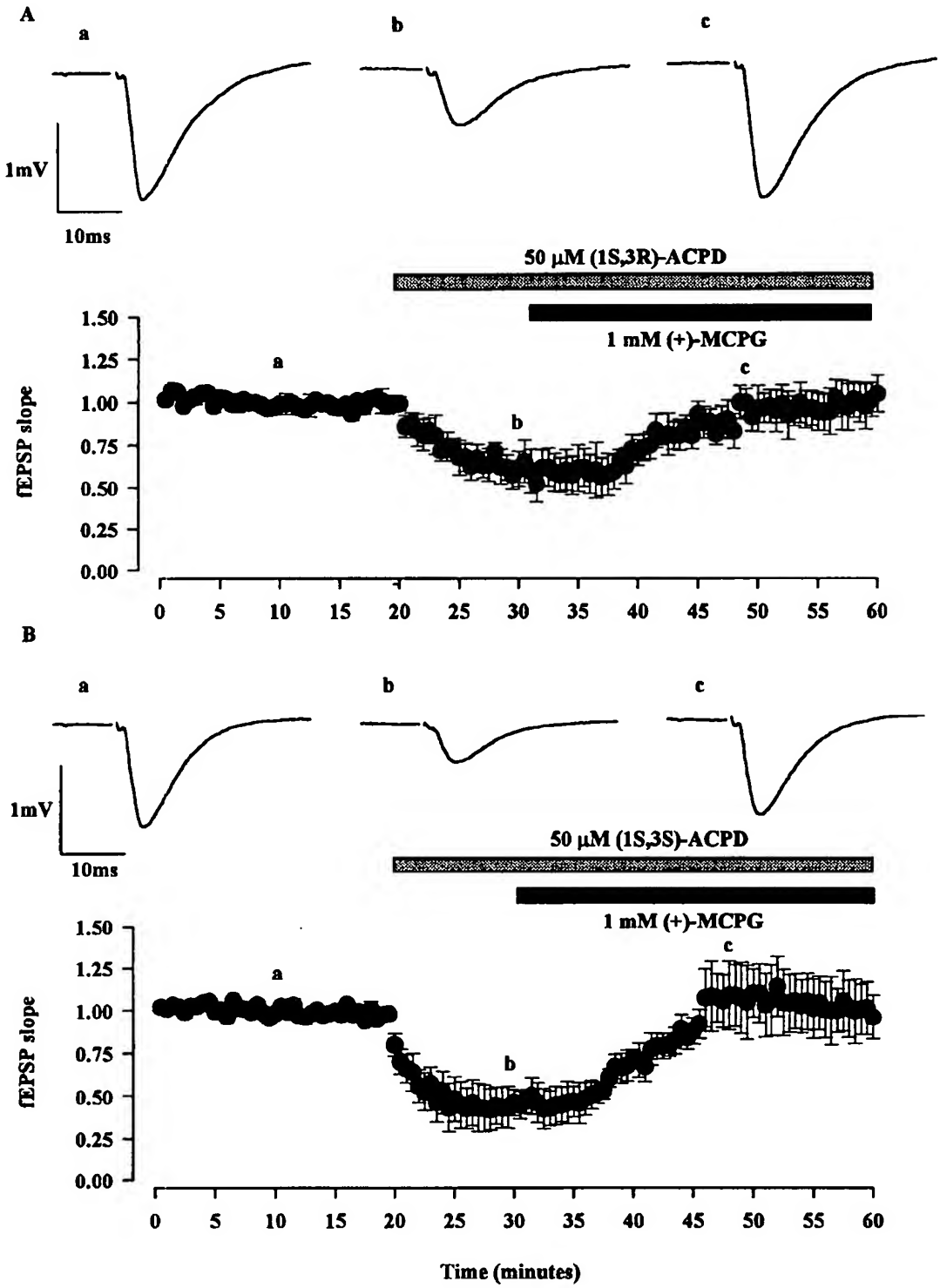

Fig. 2. Pooled data illustrating reversal of the depressant effects of (A) (1*S*,3*R*)-ACPD and (B) (1*S*,3*S*)-ACPD by (+)-MCPG. The graphs plot mean ± SEM for 4 and 3 slices, respectively, where the same protocol was adopted. (+)-MCPG (1 mM) was also tested against (1*S*,3*R*)-ACPD (50–100 μM) using a variety of different protocols and was effective in 7 of 8 slices tested.

effects of (+)-MCPG *versus* (1*S*,3*R*)-ACPD on all slices where the same protocol was tested is shown in Fig. 2(A). (+)-MCPG also reversed invariably the depressant effects of (1*S*,3*S*)-ACPD [50 μM; $n = 3$; Fig. 2(B)]. In contrast, (+)-MCPG had either a partial effect ($n = 3$) or no discernible action ($n = 3$) on depressions induced by L-AP4 (25 μM; Fig. 3). The actions of (+)-MCPG were selective towards mGluR-

mediated effects since (+)-MCPG had no effect on depressions induced by either carbachol [3–5 μM; $n = 3$; Fig. 4(A)] or (−)-baclofen [5 μM; $n = 3$; Fig. 4(B)].

Actions of MAP4

In contrast to (+)-MCPG, MAP4 (500 μM) reversed invariably the depressant actions of L-AP4 (25 μM; $n = 4$; Fig. 5). Since this is the first example of good antagonism of the actions of L-AP4 at this synapse we investigated the actions of MAP4 further, using whole-cell patch-clamp techniques. In these experiments possible indirect depressions of synaptic transmission caused by postsynaptic depolarizing actions of agonists were avoided (since the cells were dialysed with Cs^+ and voltage-clamped). In all neurones studied neither agonist tested affected holding current or input resistance. In agreement with the field potential data, MAP4 (500 μM) reversed the depression of EPSCs induced by L-AP4 (25 μM; $n = 6$; Fig. 6). In contrast, MAP4 had no effect on depressions induced by (1*S*,3*S*)-ACPD (25 μM; $n = 6$; Fig. 7). Furthermore, MAP4 was still inactive when tested at a higher concentration (1 mM) versus a lower concentration (10 μM) of (1*S*,3*S*)-ACPD ($n = 3$; data not shown).

Actions of MCCG

We next tested the effects of MCCG using whole-cell recording experiments. At a concentration of 1 mM, MCCG partially, but invariably, reversed the effects of (1*S*,3*S*)-ACPD [10 μM; $n = 8$; Fig. 8(A,C)] but had no effect on responses induced by L-AP4 [25 μM; $n = 3$; Fig. 8(B)]. The magnitude of the reversal by MCCG

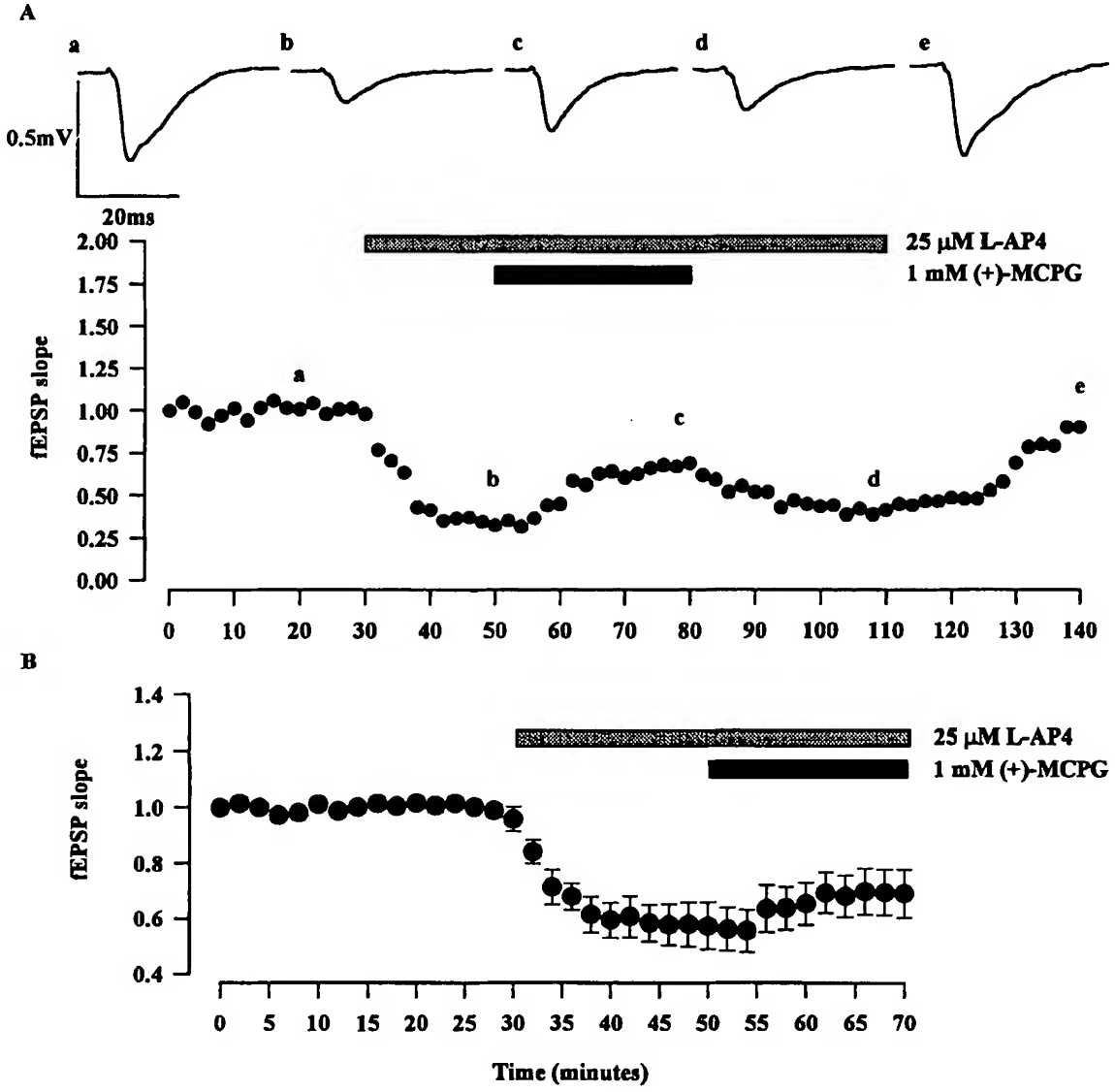

Fig. 3. Variable effects of (+)-MCPG on depressions induced by L-AP4. (A) shows an example of a small but reversible antagonism. (B) plots pooled data for all 6 slices using this protocol.

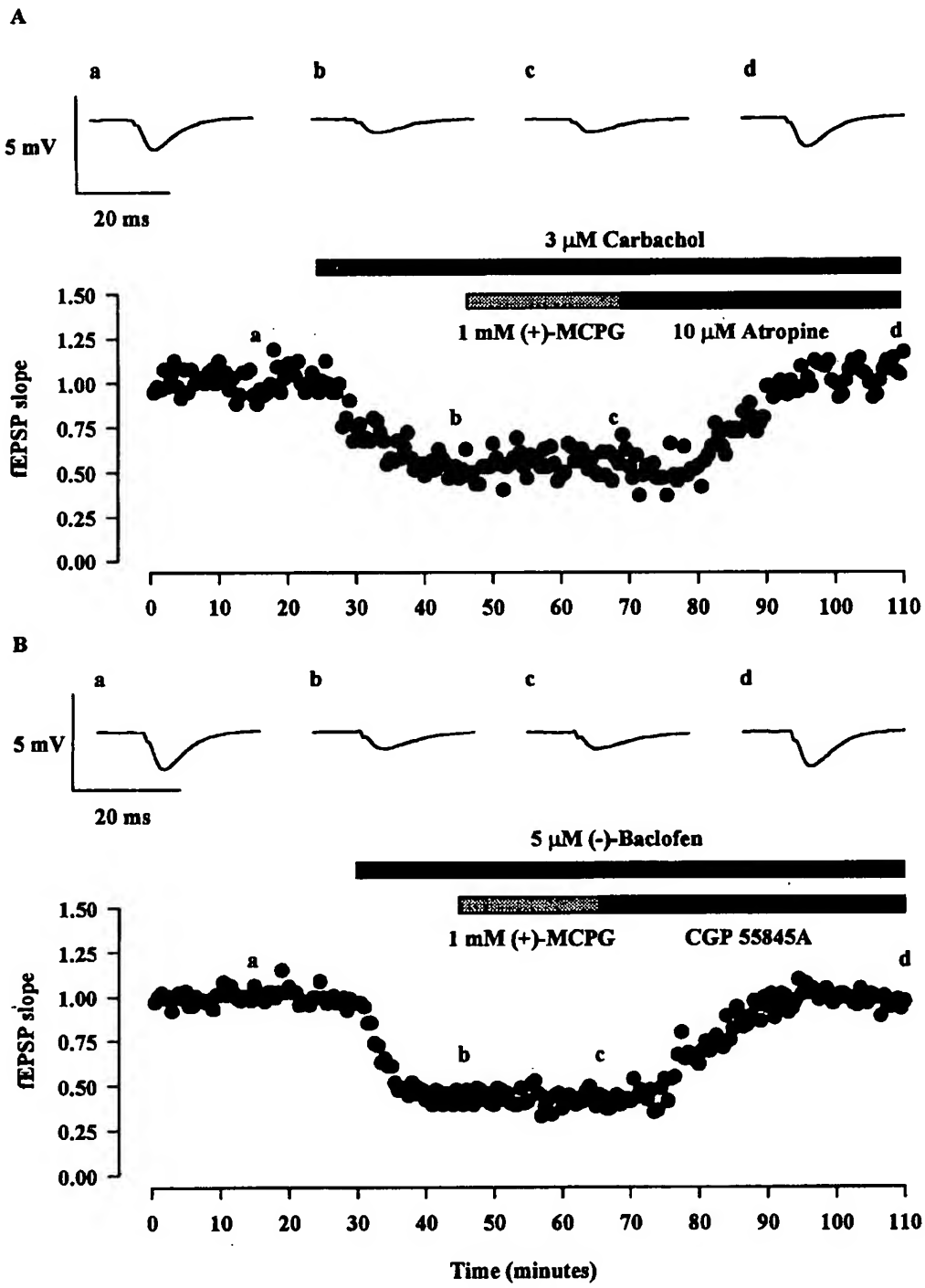

Fig. 4. Selectivity of (+)-MCPG towards mGluRs. (A) A single example showing the failure of (+)-MCPG to reverse depressions induced by carbachol. Atropine, however, reversed the depression. (B) A single example showing the failure of (+)-MCPG to reverse depressions induced by (−)-baclofen. CGP55845A (1 μM), however, reversed the depression.

was quite small but is probably an underestimate of its effectiveness as an antagonist since, in most experiments, there was incomplete recovery from the depressions induced by (1*S*,3*S*)-ACPD [Fig. 8(C)]. In contrast, the effects of L-AP4 were generally fully reversible [e.g. Fig. 5(A)].

Actions of (S)-4CPG

To complete the analysis of subgroup selective antagonists, we examined the effects of (*S*)-4CPG using both extracellular and whole-cell patch-clamp recording. The effects of (*S*)-4CPG were protocol-dependent. In field

potential recording experiments where high concentrations of agonists generated only small responses, (*S*)-4CPG (1 mM) reversed depressions induced by (1*S*,3*R*)-ACPD (50 μM; $n = 2$) and by (1*S*,3*S*)-ACPD (100 μM; $n = 4$) but had no effect on depressions induced by L-AP4 (100 μM; $n = 3$) (data not shown). In contrast, in whole-cell patch-clamp experiments where (1*S*,3*S*)-ACPD (10 μM) produced substantial depressions in voltage-clamped cells, (*S*)-4CPG had no effect [Fig. 8(C)].

DISCUSSION

The present results confirm that (1*S*,3*R*)-ACPD depresses synaptic transmission at the Schaffer collateral–commissural synapse in the rat (Harvey *et al.*, 1991; Desai *et al.*, 1992). A small part of this effect could be due to the depolarizing action of (1*S*,3*R*)-ACPD. However, a large proportion of the effect is probably presynaptic (Baskys and Malenka, 1991). The finding that L-AP4 also depresses synaptic transmission is also in agreement with previous results. Since L-AP4 has no direct excitatory action, unless the preparation is firstly "primed" with, e.g. quisqualate (Robinson *et al.*, 1986) it can be assumed that the effect of L-AP4 on field EPSPs is exclusively presynaptic (Baskys and Malenka, 1991). The depressions of EPSCs by L-AP4 can be considered to be entirely presynaptic. In contrast to a previous report (Desai *et al.*, 1992), we found (1*S*,3*S*)-ACPD to have a similar effect to (1*S*,3*R*)-ACPD. Once again a part of its effect on field EPSPs could be indirect due to its depolarizing action, but its effect on EPSCs can be assumed to be entirely presynaptic. The reason why an earlier study (Desai *et al.*,

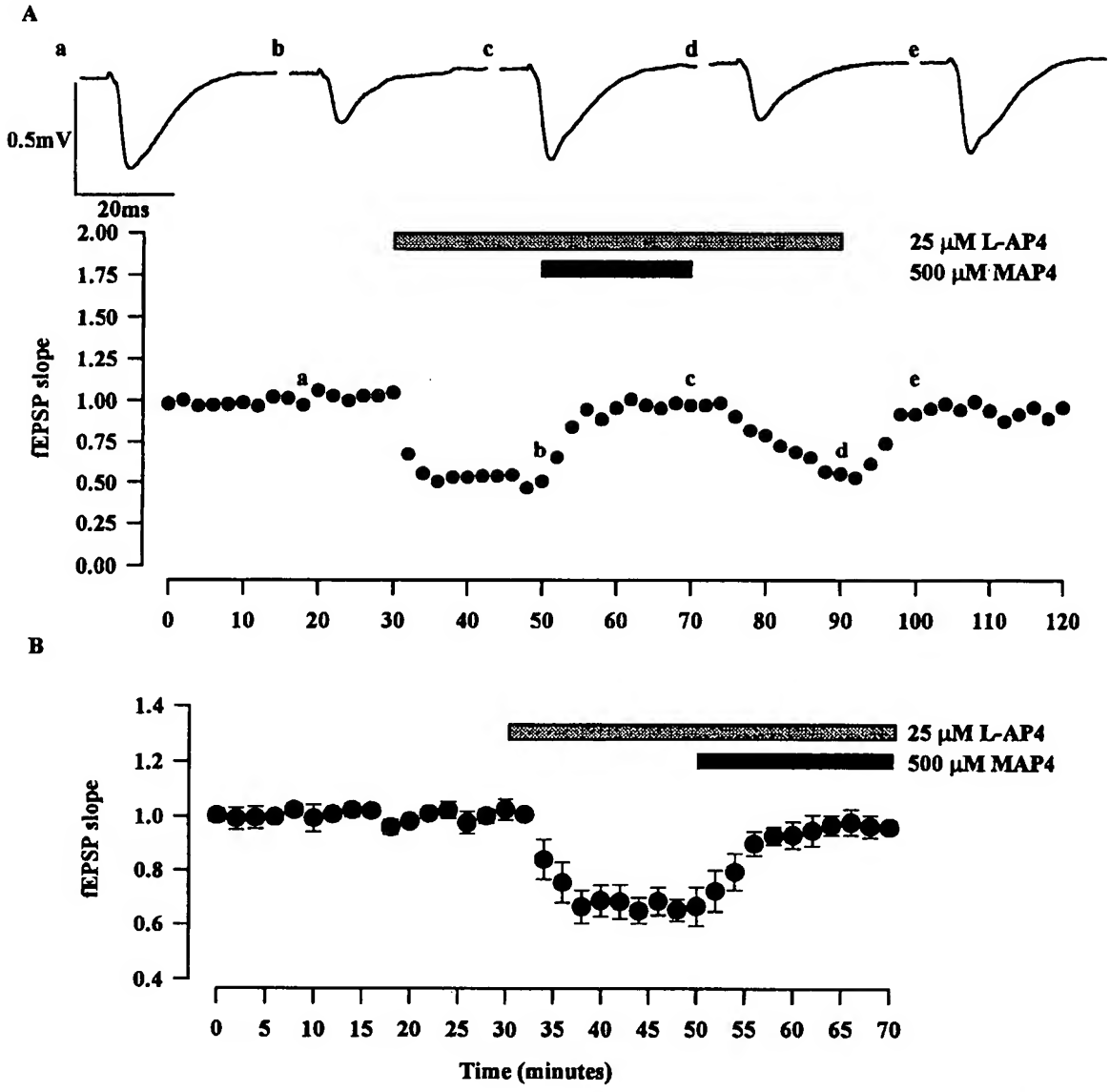

Fig. 5. MAP4 antagonizes depressions of field EPSPs induced by L-AP4. (A) Shows a single example of reversible antagonism. (B) Plots pooled data for all 4 slices using this protocol.

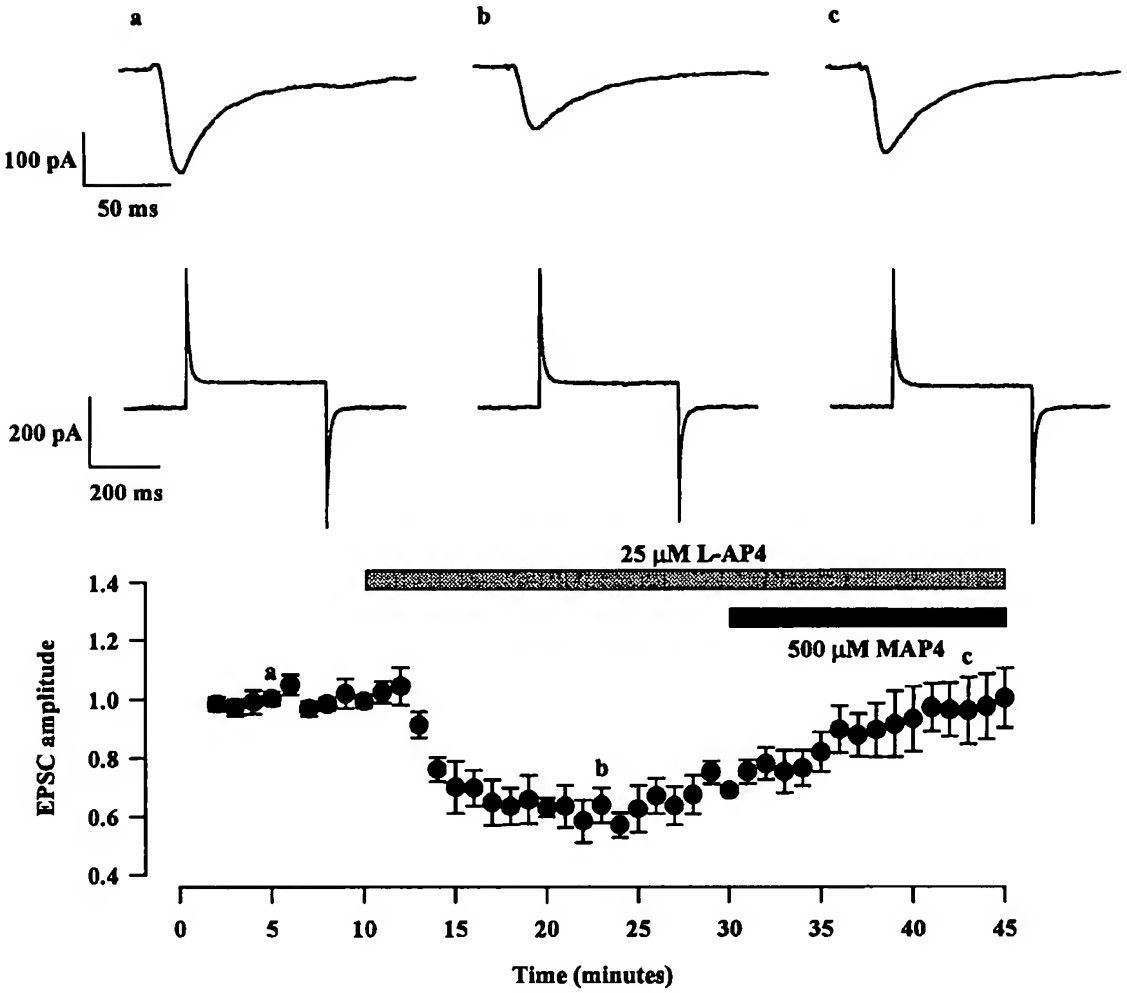

Fig. 6. MAP4 antagonizes depressions of EPSCs induced by L-AP4. The graph plots pooled data ($n = 6$) of the mean ± SEM amplitude of the EPSC plotted vs time. The traces show EPSCs (upper) and current responses to 10 mV voltage step commands (lower).

1992) failed to observe any action of (1*S*,3*S*)-ACPD is not known.

The finding that (+)-MCPG antagonized the depressant actions of (1*S*,3*R*)-ACPD extends our initial report for the Schaffer collateral–commissural synapse (Davies *et al.*, 1993). Subsequent studies in several laboratories have confirmed the effectiveness of MCPG at this site (Manzoni *et al.*, 1994; Bolshakov and Siegelbaum, 1994; Watkins and Collingridge, 1994; Selig *et al.*, 1995). However, one laboratory claimed (±) MCPG (500 μM) to be ineffective (Chinestra *et al.*, 1993) for reasons which are unclear. It is unlikely that the negative result can be attributed to the use of a 4-fold lower concentration of the effective (+) enantiomer than used in the present study, since some of the studies with positive results also used 500 μM (±)-MCPG. The present observations that (+)-MCPG does not affect the depressant actions of carbachol or baclofen, agonists for presynaptic G-protein linked cholinoceptors and GABA receptors, extends the information on the selectivity of (+)-MCPG towards mGluRs as opposed to other receptor systems (Bashir *et al.*, 1993).

MAP4 consistently reversed the effects of L-AP4. Similar antagonism of L-AP4-induced depressions has also been reported in the spinal cord (Jane *et al.*, 1994) and lateral perforant path synapse in the dentate gyrus (Bushell *et al.*, 1995). The finding that MAP4 antagonized the depressant actions of L-AP4 but not those of (1*S*,3*S*)-ACPD, while (+)-MCPG was more effective against (1*S*,3*S*)-ACPD than against L-AP4, and MCCG antagonized depressions induced by (1*S*,3*S*)-ACPD but not depressions induced by L-AP4 demonstrates the existence of two subtypes responsible for the presynaptic depressant actions of mGluR agonists at this synapse. A similar conclusion, based on antagonist studies, was made originally for the depression of monosynaptic excitation in the spinal cord (Jane *et al.*, 1994) and this principle has been extended to other synapses; inhibitory synapses in the thalamus (Salt and Eaton, 1995), the mossy fibre pathway in the hippocampus (Manzoni *et al.*,

1995) and the lateral perforant path projection in the dentate gyrus (T. J. Bushell, unpublished observations). The full subtype specificities for the antagonists used are not yet known. It is likely, however, that the effects of MCCG and MAP4 involve actions on members within subgroup II (mGluR 2 or 3) and subgroup III (mGluRs 4,6–8), respectively.

The finding that (*S*)-4CPG did not reverse depressions induced by (1*S*,3*S*)-ACPD in voltage-clamped cells is consistent with the effect of this agonist being at group II mGluRs. Indeed, (*S*)-4CPG caused a further small enhancement of the depression; an effect consistent with its agonist action at this receptor subgroup (Watkins and Collingridge, 1994). Its ability to reverse small depressions induced by higher doses of (1*S*,3*R*)- and (1*S*,3*S*)-ACPD in the extracellular experiments can be explained by an indirect effect. Thus, at these concentrations both (1*S*,3*R*)- and (1*S*,3*S*)-ACPD depolarize CA1 neurones via an action at (*S*)-4CPG-sensitive receptors (Davies *et al.*, 1995 and V. R. J. Clarke, unpublished observations); the size of the depolarizations (c. 10 mV) is sufficient to account for the small synaptic depressions (by reducing the driving force on the synaptic response). However, the possibility of presynaptic group I mGluRs, detectable under other conditions, cannot be excluded.

A paradoxical finding was that using (1*S*,3*S*)-ACPD large depressions were consistently observed in the patch-clamp experiment whereas only occasionally were large depresssions seen in the extracellular experiments. This difference was observed despite using slices obtained from the same animals for both sets of experiments, for reasons which are currently unclear. On a few occasions when large depressions of field EPSPs were observed with low concentrations of (1*S*,3*S*)-ACPD (e.g. 10 μM) the sensitivity to the slices to the group II specific agonist

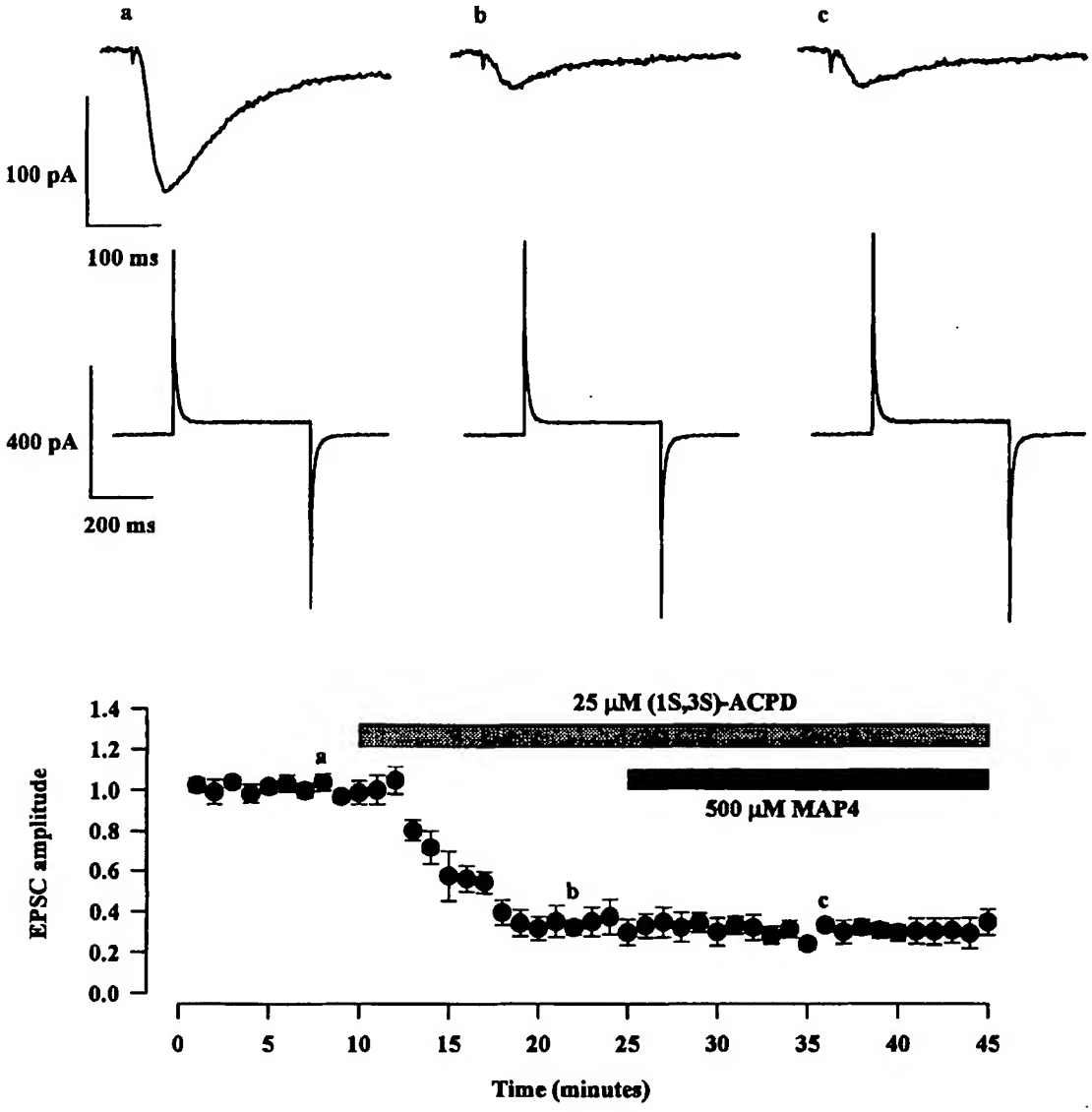

Fig. 7. MAP4 does not affect depressions of EPSCs induced by (1*S*,3*S*)-ACPD. The graph plots pooled data (n = 6).

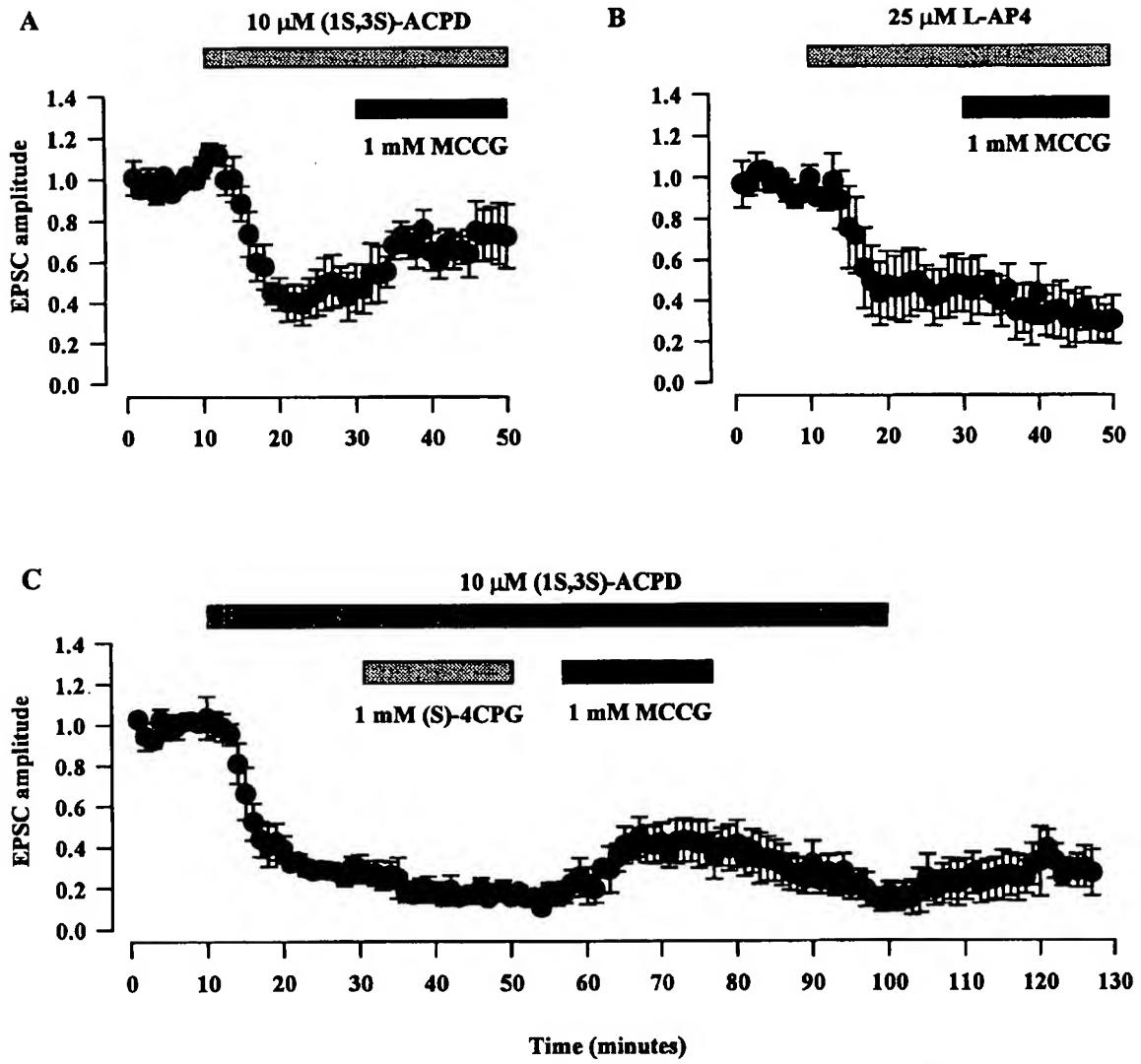

Fig. 8. MCCG reverses (1*S*,3*S*)-ACPD induced depressions. Pooled data showing the effects of MCCG on depressions induced by (A) (1*S*,3*S*)-ACPD ($n = 5$) and (B) L-AP4 ($n = 3$). In (C) are compared the effects of (*S*)-4CPG and MCCG on depressions induced by (1*S*,3*S*)-ACPD ($n = 3$). Note poor reversal of the effects of (1*S*,3*S*)-ACPD following washout of the agonist.

(2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) (Ishida *et al.*, 1993) was also tested. This agent consistently depressed synaptic transmission at 1 μM (V. R. J. Clarke, unpublished observations), a concentration which is subthreshold for activating hippocampal NMDA receptors (Wilsch *et al.*, 1994).

In conclusion, the present results provide evidence for two types of mGluRs which inhibit neurotransmitter release at the Schaffer collateral–commissural synapse and which can be selectively antagonized by MCCG and MAP4, respectively.

Acknowledgements—Supported by the MRC. V. R. J. C. is a Wellcome Trust Prize Student. M. V. is supported by the European Community (Human Capital and Mobility program).

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